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## ANNUAL REPORT

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RESEARCH ON NAVY-RELATED COMBAT  
CASUALTY CARE ISSUES, NAVY OPERATIONAL-  
RELATED INJURIES AND ILLNESSES AND  
APPROACHES TO ENHANCE NAVY/MARINE  
CORPS PERSONNEL COMBAT PERFORMANCE

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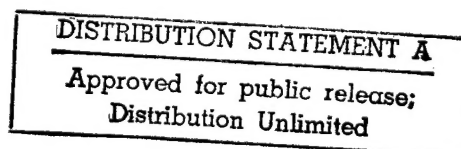
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Prepared by  
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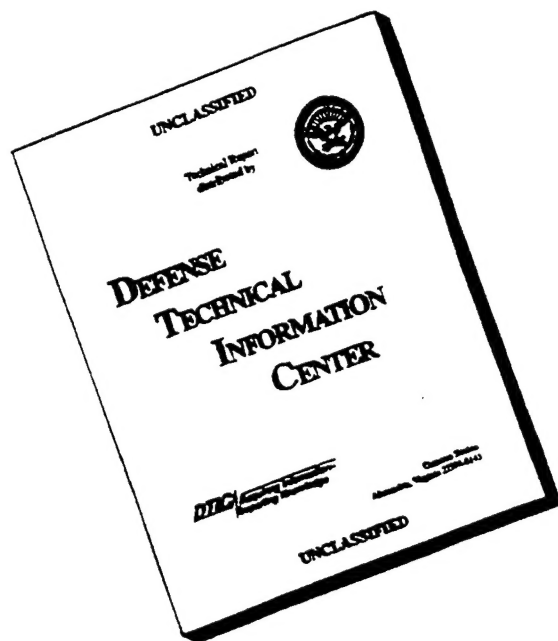
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**ANNUAL PROGRESS REPORT**  
**OPTION YEAR ONE**  
**GC-PR-2728-00**

**CONTRACT NUMBER:** N00014-95-D-0048

**REPORTING PERIOD:** December 1, 1995 - November 31, 1996

**REPORT DATE:** January 31, 1997

**RESEARCH ON NAVY-RELATED COMBAT CASUALTY CARE ISSUES,  
NAVY OPERATIONAL-RELATED INJURIES AND ILLNESSES AND  
APPROACHES TO ENHANCED NAVY/MARINE CORPS PERSONNEL  
COMBAT PERFORMANCE**

**I. INTRODUCTION**

This report summarizes the results of GEO-CENTERS' technical activities for the first option year one of the Naval Medical Research Institute (NMRI) Contract N00014-95-D-0048, Delivery Orders 002 and 003. The delivery orders encompass a variety of scientific studies that are capable of supporting ongoing and projected programs under the cognizance of NMRI; NMRI TOX/DET-Dayton, OH; NDRI-Great Lakes, IL; the NDRI Detachment-Bethesda, MD; the National Naval Medical Center-Bethesda, MD; and the U.S. Navy Clothing and Textile Facility-Natick, MA.

The format for these periodic technical progress reports consists of four sections each listed by the location of the research. The sections are (1) Descriptions of work to be performed, (2) Objectives planned for the current reporting period, (3) Summary of work performed during current reporting period, and (4) Objectives for the next reporting period. Accumulated scientific reports, technical reports and journal articles are being provided as part of this annual technical progress report. Specifically, the research conducted by GEO-CENTERS during this quarterly reporting period has been focused on the following general scientific programs:

- A. Infectious disease threat assessment and enterics programs.
- B. Immune cell biology, wound repair and artificial blood studies.
- C. Biomedical diving programs.
- D. Personnel performance enhancement programs.
- E. Breast Care Center.
- F. Dental related diseases.
- G. Toxicological studies.
- H. Human Performance and U.S. Navy Clothing Development



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## II. NMRI, Bethesda, MD

### A. INFECTIOUS DISEASE THREAT ASSESSMENT AND ENTERICS PROGRAMS

#### DESCRIPTION OF WORK TO BE PERFORMED

##### *Fernando*

- Performs research in order to develop efficient molecular assays for the detection and identification of gene sequences and strains of orthopox viruses, which can be a warfare as well as an epidemiological threat. The research involves the study of background literature on the molecular biology of these viruses and their current DNA detection methodologies, design of gene probes and tests that will identify and characterize orthopox genomic profiles, and evaluation and optimization for sensitivity, specificity and efficiency of, and finally diagnostic validation of these tests. These tests fall into three levels, namely, primary screening, secondary confirmatory and tertiary characterization.

##### *Ibrahim*

- Develop tests for the diagnosis and genetic characterization of Orthopox viruses; guide and supervise one scientist and one technician to develop long distance PCR/RFLP diagnostic procedures and determine the nucleotide sequences of selected genes of different orthopox virus species; guide and supervise one graduate student and one technician to develop molecular, probe-based colorimetric enzymatic immunoassays (EIA) for the detection and specific identification of *Yersinia pestis* and *Bacillus anthracis*.

##### *Jendrek*

- Conducts fermentations in a BL-3 suite and depending on the organism of the fermentation may also perform some or all of the downstream processing associated with the project. He also creates all associated paperwork (standard operating procedures, batch records, etc.) with the fermenter and related equipment. Scott also does much of the HPLC work towards optimizing current protein purification methods and procedures, as well as all of the molecular biology associated with his position.



*Kerby*

- Senior Scientist; develop diagnostic systems to detect and differentiate Orthopox viruses

*Pifat*

- Senior Scientist II
- Contractor Support for the establishment of USAMRIID's GLP capabilities

*Weeks*

- Serve as an associate of the principal investigator for a research program involving pathogenic, molecular, and biochemical analysis of bacteria and their virulence factors. Experimentation requires knowledge and proficiency of laboratory techniques and procedures for performing biochemical and immunological analyses. Conducts surveys of the scientific literature to develop background data on techniques and formulates approaches for the investigations, develops experimental protocols, defines the objectives and priorities of subsidiary problems and arranges the details of cooperative investigations with other organizations when necessary. Is responsible for the general administration of the laboratory reagents, solutions, enzymes, and other materials and equipment used in conducting the studies described. Is responsible for the cleanliness and orderliness of working areas, freezers, and refrigerators. Is responsible for the training and orientation of all new laboratory technicians. Organizes and accumulates repositories of bacterial strains, plasmids, enzymes and sera with sufficient documentation of the histories of each. Maintains sufficient stocks of all reagents, supplies, and equipment required for a well organized molecular biology laboratory. Performs other duties as assigned. Immunizations are required.

**TECHNICAL OBJECTIVES FOR THIS REPORTING PERIOD**

*Fernando*

- Will continue with the secondary level DNA PCR tests, developing new 20 kb segment amplifications of the remainder of orthopox viral genome.



- Will also explore new high resolution electrophoretic procedures for RFLP DNA fragment analysis in order to improve the efficiency of DNA fingerprinting of orthopox viruses.

*Ibrahim*

- Complete testing of oligonucleotide primers for all 7 genes.
- Complete testing of fluorogenic probes for 3 genes.
- Analyze entire sequences of the 7 selected genes. Re-evaluate probe design.
- Start testing variola DNA with the 5' nuclease assay.
- Complete at least one manuscript.

*Jendrek*

- Will show if the plasmid SJ4 in *B. subtilis* makes protective antigen in amounts comparable to *B. anthracis* and/or greater than pMK3. Scott will start to ferment *E. coli* in order to produce F1, an immunogenic protein from *Y. pestis*. He will create a protocol for the fermentation and purification of F1, for which he will create batch records and SOP's.

*Kerby*

- Design and synthesize PCR primers from the published sequences of Variola (VAR), and Vaccinia (VAC), that will be cross-reactive with Camelpox (CML), Monkeypox (MPV), and Cowpox (CPV).
- Sequence the resulting amplified PCR products from the above tested primers to add to our sequence databank .
- To have tested and have available at least one set of diagnostic primers for the amplification of : 1) DNA polymerase gene, 2) Thymidine Kinase gene, 3) RNA polymerase - 147 kDa. gene, 4) RNA polymerase - 132 kDa. gene, 5) Hemagglutinin gene, 6) Interferon- $\gamma$  Receptor Homology gene, and 7) Tumor Necrosis Factor Homology gene for VAC, CML, MPV, and CPV.

*Pifat*

- Assist in developing pertinent SOP's and other regulatory documentation
- Assist in developing validation plans for relevant bio-assays
- Assist in converting basic research laboratories into GLP-compliant laboratories



- Assist in selecting and establishing training courses and seminars to enhance USAMRIID's general knowledge of regulatory compliance issues.

*Weeks*

- The objective for this year was to finish the work on the Mud mutagenesis of the pFra plasmid of *Yersinia pestis* and prepare a manuscript for publication. The other objective for this period was to start work with V antigen and define its affects on human immune cells.

**SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING PERIOD**

*Fernando*

- Optimized an improved DNA labeling procedure, which labels the DNA fragment ends with a fluorescent dideoxy-NTP moiety by using deoxynucleotidyl terminal transferase.
- Started developing amplification primer sets for the rest of the viral genome, by arbitrarily dividing the genome of the reference orthopox virus, Vaccinia, into 8 overlapping "20 kb" segments, which ranged from 15-26 kbp in size, the segment already tested being the 3rd. The ends of these Vaccinia genomic segments are highly homologous to the corresponding regions in Variola (Smallpox), the basis for universal orthopox-specificity. Primer sets for the new segments were designed as before using the "Oligo" software and synthesized; then screened these primers against the four reference orthopox species DNAs. Primer sets for 3 segments (2nd, 5th & 6th) were found to successfully amplify all four references species. The other primer sets did not amplify every one of the four species except Vaccinia, and therefore must be redesigned. The four successful primer sets cover the amplification of 60% of the genomes. RFLP profiles of the new segments were also performed. He is currently evaluating a precast acrylamide gel mini-electrophoresis system based on simple fluorescent dye staining of DNA bands.

*Ibrahim*

- Designed over 60 sets of PCR primers for amplification of selected fragment of orthopoxvirus genes. Supervised the testing and evaluation of the primers. Tested 35 sets of primers against four different species of orthopoxviruses.



- Designed and tested two fluorogenic probes for cowpoxvirus and monkeypoxvirus.
- Designed new probes for Variola virus.
- Supervised the sequencing of 7 genes of three orthopoxviruses.
- Coordinated through CDC the acquisition of selected fragments of the variola genome for testing at USAMRIID.

Publications/Abstracts

- Ibrahim, M. S., Esposito, J. J., Jahrling, P. B. and Lofts, R. S. (1996). The potential of 5' nuclease PCR for detecting a single-base polymorphism in orthopoxviruses. Molecular and Cellular Probes (submitted).
- Ibrahim, M. S., Ezzell, J. W. and Henschel, E. A. (1996). Chemiluminescence detection of PCR-amplified sandfly fever Sicilian virus RNA. Third Annual Novel Amplification Technologies, Washington, D.C., Dec. 16-17, 1996.

*Jendrek*

- At the current time the shuttle vector is not going into *B. subtilis* WB600 at all, he will continue to try to get this to work. He grew F1 producing *E. coli* and purified the F1 according to current protocols. He conducted PPA102CR4 fermentations for the production of PA to be used in animal tests and he finalized and updated the batch record for that particular process.

*Kerby*

- Designed, synthesized, and tested 34 sets of primers for the seven genes.
- Fourteen sets successfully amplified, all four reference viral strains which included the DNA polymerase gene, both RNA polymerase genes, the Hemagglutinin gene, and the Tumor Necrosis Factor Homology gene. Primer sets for the Thymidine Kinase gene were successful for only CML and CPV reference viral strains while primer sets for the Interferon  $\gamma$  Receptor Homology gene were successful for VAC, CML, and CPV only.
- Sequenced the largest PCR product from a representative of each of the seven genes and added to our database.



*Pifat*

- Reviewed all existing SOP's at USAMRIID and written a number of others. These SOP's are currently in the Quality Assurance review process and ready to be implemented. Other documentation systems continued to be implemented such as: a receipt and qualification system for Reference Materials; a buffer preparation scheme and lot numbering system; a protocol review log; communication records; equipment maintenance and repair records. A number of Study Specific Procedures were written, dealing with upcoming products, their quantitation and their qualification.
- Validation Protocol for one of the Anthrax ELISAs was modified to take into account available reagents and historical data. The GLP-compliant validation process is scheduled to take place in January 1997. Other validation protocols for Anthrax and Venezuelan Equine Encephalitis (VEE) virus have also been initiated.
- Continued to have planning meetings dealing with basic research laboratories to be converted to GLP-compliant laboratories. Renovations have been planned and equipment and reagents have been ordered.
- Participate in the organization of four training courses for USAMRIID personnel which have dealt with: 1) basic GLP regulations, 2) SOP writing, 3) GLP's for analytical procedures, and 4) GLP's as they apply to Study Directors.

Publications, Abstracts, Presentations, Speeches

- Participated in monthly meetings with the SAIC/NCI for the GMP production of recombinant Anthrax PA.
- Participated in organizational meetings on the development of vaccines against Botulinum toxin and the validation of related assays.
- Participated in organizational meetings on the development of vaccines against Staphylococcus Enterotoxin B (SEB) and the validation of related assays.
- Attended a one day workshop on Quality Assurance and Data Management (9/18/96)
- Attended the regional NCAB/AALAS meeting dealing with the humane care and use of laboratory animals (9/19/96)
- Attended a seminar on new regulations governing the export and import of etiologic agents (10/2/96)
- Attended the annual meeting of the American Society of Quality Assurance (10/14/96 to 10/17/96)
- Attended a meeting with representatives from USAMMDA and Perimmune Inc. dealing with the development of therapeutics against botulinum toxin and assay validation (11/13/96) and another meeting with USAMRIID representatives and Perimmune Inc. (12/9/96).





- Attended a meeting with representatives from USAMMDA and Batelle Inc. dealing with botulinum toxin and assay validation.
- Attended a meeting at the Joint Program's Office (JPO) related to funding and product development for the defense against potential Biological Warfare agents (11/15/96).
- Assembled a briefing for Gen. Doesburg (JPO) on recent advances in the development of a safe and efficacious vaccine against VEE virus.

*Weeks*

- Successfully completed the first attempt at purifying the V antigen protein prepared by PerImmune.

**GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD**

*Fernando*

- Continue with experiments on the other genome segments and the mini-gel system for orthopox DNA fingerprinting

*Ibrahim*

- N/A

*Jendrek*

- Will develop an HIC step for the current purification scheme of protective antigen. Will write an updated protocol for the production of F1 and its purification to increase the stocks on hand and the purity of those stocks. He will continue with his current molecular biology projects and also make a producer of F1 in which the plasmid is stable over time. He will also create a new protocol for the production and purification of F1.

*Kerby*

- Design, synthesize, and test more primers so that all seven genes will have primers that can amplify successfully all four reference viral strains.
- Using the newly identified sequences; design, synthesize, and test primers so that the complete gene sequence can be obtained from all seven genes.



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- Validate the new sequences.
- Establish protocols and procedures that have been optimized for rapid PCR amplification, rapid gel electrophoresis, and automated sequencing.

*Pifat*

- Assist in developing pertinent SOP's and other regulatory documentation
- Assist in developing Validation plans for relevant bio-assays
- Assist in converting basic research laboratories into GLP-compliant laboratories
- Assist in selecting and establishing training courses and seminars to enhance USAMRIID's general knowledge of regulatory compliance issues.

*Weeks*

- The objectives for next quarter are to continue the purification of V antigen in order to begin animal experiments, finish the work on the pFra plasmid and prepare a manuscript for publication.



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## **II. NMRI, Bethesda, MD**

### **B. IMMUNE CELL BIOLOGY, WOUND REPAIR RESEARCH AND ARTIFICIAL BLOOD PROGRAM**

#### **DESCRIPTION OF WORK TO BE PERFORMED**

##### *Chavez*

- Principal Investigator, Blood Research Detachment - Performs basic research on the physical properties of hemoglobin and hemoglobin-based blood substitutes. Hemoglobin is the protein responsible for oxygen transport. Hemoglobin oxidation, heme stability within hemoglobin, and nitric oxide interaction with the red cell membrane are the major focus areas at this time.
- Scientific Advisor, Pilot Plant Facility - Provides scientific consulting on a variety of pertinent operations including analytical assay development, hemoglobin biochemistry, and protein purification and stabilization.

##### *Christian, Wohlrabe*

- Provide assistance to the adenovirus surveillance project within the Preventive Medicine division of Naval Hospital, Great Lakes, IL.
- Assist in the development and implementation of "Operation Stop Cough", a programmatic approach to reducing respiratory illness among Navy recruits.

##### *Kidwell*

- Review and summarize accounting reports from NMRI, GEO-Centers, USUHS, and the Henry Jackson Foundation. Prepare and track required paperwork, when necessary, to transfer funds between accounts.
- Coordinate preparations for conferences including formatting and submitting abstracts, submitting registration fees, coordination of art work for posters, preparing travel request forms, obtaining clearance for visits to foreign countries, making travel arrangements and hotel reservations, and preparing travel reimbursement forms.
- Assist in the preparation of manuscripts including editing the text, formatting the document to comply with specific journal requirements, verifying all references via MedLine, preparing the necessary number of copies and other documentation to be included, and submitting the manuscript to the journal. Track the status of all



manuscripts from first draft to final publication and receipt of reprints. Respond to reprint requests for all manuscripts prepared by the Resuscitative Medicine Program.

- Process and track purchase requisitions for the department in the absence of the Supply Counterpart.
- Assist in the preparation of annual reports, proposals, etc. by compiling budgetary information, formatting and typing the documents, and submitting to the proper personnel.
- Compile and submit a weekly departmental MED-02 report to the Scientific Director. This report includes items such as presentations given by resuscitative Medicine staff, notable events, distinguished visitors, and publications.
- Compile and submit to the Commanding Officer a Command Historical Report for the Resuscitative Medicine Program. This report includes all presentations, notable events, distinguished visitors, and publications for the year.
- Assist in the preparation of animal use protocols. Track all animal use and animal per diem reports for the Resuscitative Medicine Program to ensure that sufficient funds are available for each protocol.
- Work with GEO-Centers to coordinate a scientific conference. This includes reviewing site arrangements, compiling addresses for attendees, preparing letters and invitations to attendees, preparing schedules and cost estimates, coordinating responses of invited speakers, making arrangements for technical equipment, recording the proceedings, and preparing the transcripts.
- Coordinate seminars given by in-house scientists or visiting scientists including location, meals, lodging, and travel arrangements.
- Provide general administrative support including writing, editing, and typing letters and memos, copying, filing, faxing, etc.
- Perform MedLine searches and obtain copies of pertinent articles.
- Assist in the preparation of Standard Operating Procedures (SOPs) for the Resuscitative Medicine Program and maintain a central file of all SOPs for the department.
- Maintain Material Safety Data Sheet collection.



## TECHNICAL OBJECTIVES FOR THIS REPORTING PERIOD

### *Chavez*

- The following abstract was presented: "Heme Stability of Partially Oxidized Hemoglobins," M.D. Chávez, B. E. Shrader, H.S. Zahwa, and V.W. Macdonald, 1996 Biophysical Meeting, February 17-21, Baltimore, MD.
- Heme affinity experiments using rapid scanning spectroscopy have been completed.
- Heme exchange experiments have been completed.
- The poster "An Improved Process for the Production of Sterile Modified Hemoglobin Solutions" was presented at the 1996 Current Issues in Blood Research and Development in San Diego, CA.
- Final development of the large scale A<sub>0</sub> production.
- Submission of the pilot plant manuscript
- Submission of the liposome-encapsulated hemoglobin manuscript
- Initiation of nitric oxide - hemoglobin binding experiments
- Submission of abstract for the 1997 Biophysical Society Meeting

### *Christian, Wohlrabe*

- Implement culture surveillance for adenoviral illness among recruits.
- Develop contacts and become familiar with Navy health care services for recruits, to assist in development and coordination of Operation Stop Cough.
- Liaison between investigators, providers, laboratory staff, and patients to provide adenovirus surveillance information.

### *Kidwell*

- Coordinate arrangements with GEO-Centers for the "Cell Biology of Hypoxia" conference scheduled in September 1996.
- Establish a budget tracking system for FY 96 and prepare monthly budget spreadsheets.
- Prepare budget information for FY 97 and re-calculate proposals as necessary to reflect the change in the way NMRI overhead is calculated in the new fiscal year.
- Assist the Wound Repair Enhancement personnel in making the transition to the new organization structure as smooth as possible.
- Provide support in the on-going areas such as budget reports, conferences, manuscripts, animal use protocols, general administrative duties, etc.



## SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING PERIOD

*Chavez*

- Presentation of the poster at the 1996 Biophysical Society went well. The most enlightening result of the work was that an increased population of liganded ferrous (reduced) heme within a partially oxidized  $\alpha\alpha$ -cross linked hemoglobin tetramer increases the rate of loss of the oxidized heme. This is further evidence that the liganded ferrous  $\alpha\alpha$ -cross linked hemoglobin exists in an intermediate destabilized configuration. To our surprise, anaerobic conditions increased the rate of heme exchange in partially oxidized hemoglobin. Oxidation of the ferrous deoxy-heme is accelerated under experimental conditions, leading to the increased heme exchange. Free heme is well known to catalyze various peroxide compounds that are toxic and undesired. The experiments have been completed and manuscript preparation is underway.
- Heme affinity experiments have been completed and manuscript preparation is underway. In doing the heme extraction procedure, it was observed that heat treated, flash photolyzed hemoglobin A<sub>0</sub> was less stable than hemoglobin A<sub>0</sub> not subjected to this treatment. In discussion with other researchers and collaborators, this observation has been noted in their studies as well. At this time, investigation relating to this observation is not being pursued.
- We have submitted the manuscript "An Improved Process for the Production of Sterile Modified Hemoglobin Solutions" to the journal Biologicals. This journal was chosen because it emphasizes biotechnology papers and it can be found on Medline. The results were also presented at the 1996 Current Issues in Blood Substitute Research and Development.
- The 1996 Current Issues in Blood Research and Development Course went as expected. Several companies (Somatogen, Northfield, Alliance, Hemosol, Baxter, etc.) are pressing forward toward Phase III trials of their respective products. Of note, the companies are targeting the use of their products for specific purposes (ex. irradiation of tumors), not as a "general" blood substitute at this time. Toxicity issues remain as significant obstacles in final approval, including the topics of heme exchange and loss, oxidation, and nitric oxide interactions. I remain convinced that these issues are worth investigating to understand the mechanisms of hemoglobin and develop better products in the future. On the scientific side, more evidence



was presented that suggests that carbon monoxide, like nitric oxide, can also act as a neurotransmitter.

- We have submitted the manuscript "Liposome Encapsulation Attenuates Hemoglobin-Induced Vasoconstriction in Rabbit Arterial Segment" to the Journal of Applied Physiology. We have made minor corrections and resubmission has already taken place.
- Two summer students, Brooke Shrader and Janis Sanders, have interned in my laboratory June 17-August 8. Their project involves a current hot topic in physiology, nitric oxide. Nitric oxide is a vasodilator, however, the exact mechanism of its contribution to the regulation of blood pressure is not known. Our study is focusing on the interaction of nitric oxide with red blood cells. Preliminary results went as expected, with the nitric oxide interaction being much slower with intracellular hemoglobin (red blood cells) versus extracellular hemoglobin. Further experiments are underway to determine the interaction between the red cell membrane and nitric oxide.
- The manufacturing of hemoglobin A<sub>0</sub> on a large scale ran into several obstacles that ultimately suspended the project. Upon preparing the ion exchange columns, the CM-52 material compressed too much, causing the column pressure to exceed recommended values. After calling technical support, it was found that initial information provided to me was incorrect and the column size used was too long/narrow for the application. In addition, endotoxin levels for this column were unacceptable. A procedure to remove pyrogens was successfully developed and the CM-52 column was repoured at a shorter length to alleviate pressure buildups. The large scale preparation began but was unceremoniously cut off by a cracked peristaltic pump tubing used. Although I feel the procedure is now in place and viable, the large scale hemoglobin A<sub>0</sub> manufacturing was terminated due to the time constraints caused by the closure of the pilot plant.
- The abstract "Enthalpy Changes of the Step-wise Ligation of Hemoglobin" has been submitted for the 1997 Biophysical Society Meeting.

Presentations, Publication, Abstracts, etc.

- "Heme Stability of Partially Oxidized Hemoglobins" M.D. Chávez, B.E. Shrader, H.S. Zahwa, and V.W. Macdonald, Biophys. J., 1996, 70(2), 218a.
- "In Improved Process for the Production of Sterile Modified Hemoglobin Solutions" M.D. Chávez<sup>1</sup>, F.A. Highsmith<sup>2</sup>, C.M. Driscoll<sup>2</sup>, B.C. Chung<sup>2</sup>, V.W. Macdonald<sup>1</sup>, J.M. Manning<sup>3</sup>, L.E. Lippert<sup>2</sup>, R.L. Berger<sup>2</sup>, and J.R. Hess<sup>2</sup>, presented at the 1996 Current Issues in Blood Substitute Research and Development in San Diego, CA, March 17-20. <sup>1</sup>Blood Research Detachment, Walter Reed Army



Institute of Research; <sup>2</sup>Bionetics Corporation, Rockville, MD; <sup>3</sup>The Rockefeller University, New York, NY.

- "An Improved Process for the Production of Sterile Modified Hemoglobin Solutions" F.A. Highsmith<sup>2</sup>, C.M. Driscoll<sup>2</sup>, B.C. Chung<sup>2</sup>, M.D. Chávez<sup>1</sup>, V.W. Macdonald<sup>1</sup>, J.M. Manning<sup>3</sup>, L.E. Lippert<sup>2</sup>, R.L. Berger<sup>2</sup>, and J.R. Hess<sup>2</sup>, Biologicals, submitted. <sup>1</sup>Blood Research Detachment, Walter Reed Army Institute of Research; <sup>2</sup>Bionetics Corporation, Rockville, MD; <sup>3</sup>The Rockefeller University, New York, NY.
- "Liposome Encapsulation attenuates Hemoglobin-Induced Vasoconstriction in Rabbit Arterial Segment" A.S. Rudolph<sup>1</sup>, A. Sulpizio<sup>2</sup>, P. Hieble<sup>2</sup>, V. M. Macdonald<sup>3</sup>, M.D. Chavez<sup>3</sup>, and G. Feuerstein<sup>2</sup>, J. Appl. Physiol., submitted. <sup>1</sup>Center for Bio/Molecular Science and Engineering, Code 6910, Naval Research Laboratory, Washington, D.C. 20375-5348; <sup>2</sup>Dept. of Cardiovascular Pharmacology, SmithKline Beecham, King of Prussia, PA 19406; <sup>3</sup>Blood Research Detachment, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100
- "Enthalpy Changes of the Step-wise Ligation of Hemoglobin" J.A. Foltz, K. Franklin, H.S. Zahwa, M.D. Chávez, and R.L. Berger, submitted for the 1997 Biophysical Society Meeting.

*Christian, Wohlrabe*

- Arranged storage, distribution, and collection of culture materials for adenovirus surveillance.
- Assisted in training providers and laboratory staff on culturing.
- Reviewed records and provided quality control for case reports of respiratory illness.
- Initiated tracking of cultures and case reports for adenoviral surveillance.
- Completed training, and began assisting in the direct collection of cultures.
- Arranged monthly mail-out of culture materials to Naval Health Research Center.
- Initiated inspections of hand washing facilities for recruits, as a quality metric for Operation Stop Cough.
- Initiated occupational health evaluations and annual training, as new health care workers.





*Kidwell*

- Edited/formatted 16 manuscripts for submission to scientific journals. Of these 16 manuscripts, seven are in final review/clearance at NMRI, two have been submitted and are being reviewed by the journals, four are "in press", two have been published, and one was returned for further data.
- Edited/formatted one "Letter to the Editor" which was published in *Transplantation*.
- Created and maintained a manuscript tracking database which indicates the status of all manuscripts which have come across my desk.
- Created a filing system for manuscript drafts, figures, photos, final copies, and reprints.
- Responded to all internal and external reprint requests for recently published papers.
- Created and maintained a detailed budget spreadsheet to track and manage all Wound Repair accounts to include salaries, overhead charges, animal per diem, supplies, travel and transfers. The spreadsheets are updated on a monthly basis and reports are provided to the principal investigators.
- Transferred funds as necessary to ensure proper balance of all accounts.
- Prepared a budget report for CDR Yaffe on a monthly basis.
- Coordinated the visit/seminar of two speakers.
- Worked with Lisa Dalton of GEO-CENTERS to coordinate the "Cell Biology of Hypoxia, 1996" conference held in Gaithersburg and had an attendance of approximately 50 people. Recorded the conference and prepared and published transcripts (See Section V).
- Assisted in the preparation of four grant proposals, of two DARPA pre-proposals, nine work unit annual reports and four work unit termination reports.
- Assisted in the preparation of two animal use protocols and established an animal use tracking system.
- Assisted in the preparation of Standard Operating Procedures. This included compiling MSDS's for all chemicals and completing Hazardous Materials forms. Created a central SOP filing system.
- Provided back-up order processing and tracking support.
- Maintained the Material Safety Data Sheet collection.
- Assisted in the preparation of personnel performance appraisals and standards.
- Prepared a departmental MED02 report for the Scientific Director highlighting items of interest on a weekly basis.
- Compiled all weekly MED02 reports to create a departmental Command Historical Report to be submitted to the Commanding Officer.
- Requested and obtained approval for a foreign visiting scientist to work at NMRI.



- Prepared four abstract for presentation to the Surgeon General.
- Began assisting the new Program Head, Dr. Richard McCarron, in settling in to his new position and becoming organized.
- Provide general administrative services to the department including typing letters and memos, copying, filing, distribution of mail, etc. as needed.
- Act as travel liaison for the department. This involves requesting country clearances, making travel arrangements, and completing reimbursement forms for all departmental personnel.
- Made all necessary arrangements for investigators to attend eight domestic scientific conferences. This included preparing and submitting abstracts, registering participants, making all travel and accommodation arrangements, and preparing and submitting reimbursement forms.
- Made all necessary arrangement for investigators to attend three international scientific conferences. This included requesting country clearances, assisting with visas applications, preparing and submitting abstracts, registering participants, making all travel and accommodation arrangements, and preparing and submitting reimbursement forms.

Presentations, Publication, Abstract, etc.

- Cell Biology of Hypoxia, 1996. TB Nielsen and JL Kidwell (Eds). NMRI Technical Report, September 1996.

**GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD**

*Chavez*

- Closure of the plant pilot facilities has caused a major shift of specific priorities. Research concerning the blood substitute field has ceased, and current research is now focused on the structure and function of red cell membranes. In short, a thorough understanding of red cell membrane function is necessary to impede or prevent cell degradation, rigidity, and fragmentation. Blood is a valuable resource to the U.S. Army; currently, the use of this precious resource is very inefficient due to remote locales, storage requirements, transportation, etc. The projects listed below are being initiated to study the red cell aging problem in hopes of extending the shelf life of blood, a primary mission goal.
- Thiol reactivity - the formation of disulfide bridges through thiol groups within the membrane proteins is thought to cause cell rigidity in erythrocytes (red blood cells).



Our study will monitor the integrity of the thiol groups as a function of aging to find any possible correlation.

- Nitric oxide interactions with erythrocytes - Nitric oxide is known to cause vasodilation. Nitrosothiols, formed between nitric oxide and thiol groups, are also potent vasodilators. Our experiments will show whether nitric oxide reactivity is preserved through the formation of nitrosothiols on the erythrocyte membrane.

*Christian, Wohlrabe*

- Continue work on the adenovirus surveillance project, modifying procedures as the protocol changes.
- Continue to provide data on hygiene and hand washing, as metrics for Operation Stop Cough.
- Assist with the preparation of a poster presentation on Operation Stop Cough for the Navy Environmental Health Center Workshop in February 1997.
- Assist with the development of a protocol for investigating the value of air quality enhancements (ultraviolet light air cleaners) for reducing respiratory illness among recruits.
- Complete occupational health evaluations and annual training requirements for health care workers.

*Kidwell*

- Continue providing support in the on-going areas such as budget reports, conferences, manuscripts, animal use protocols, general administrative duties, etc.



## II. NMRI, Bethesda, MD

### C. BIOMEDICAL DIVING RESEARCH

#### DESCRIPTION OF WORK TO BE PERFORMED

##### *Lee*

- Scientist I and representative for GEO-CENTERS for the NMRI contract. Serves as and performs work as a research assistant. Responsible for implementing and carrying out aspects of the Navy Blood Storage Project being conducted at the University of New Mexico (UNM).

##### *Shea*

##### Alzheimer Project:

- To perform microdialysis experiments in the CNS of rats which have previously been lesioned at the nucleus basalis Mynert (NBM) via the drug NMDA.
- Analyze the neurotransmitters acetylcholine (ACh), norepinephrine (NE), and serotonin (5-HT), in microdialysis perfusate obtained from the above experiments.

##### Oxygen toxicity Project:

- Run trial microdialysis experiments in the newly designed hyperbaric chambers prepared for 100% oxygen environment under deep dive conditions.

##### Dityrosine Project:

- This is a joint project with Dr. Ajay Verma in the department of Uniform Serv. Univ. Hlth. Sci. to establish an assay for dityrosine and to determine if there is a biological response in this compound after various stressors to the animal. We will also look for this under certain human pathological conditions.

##### *Obowa*

- Provide technical assistance in the Diving Medicine research laboratory investigating exposure to hyperbaric oxygen (HBO) and its effects on the CNS. Prepare brain tissues for staining, section tissues using the cryostat, perform immunohistochemical staining methods on tissue sections, care for animals, perform surgical procedures on rats, and order laboratory equipment and supplies.



*Porter*

- To support in the selection and testing of a hyperbaric CO2 analyzer for fleet submarine dry deck shelter use.
- To support analysis of fleet soda lime for possible contamination and to analyze the samples for specific dye concentrations when indicated.
- To assist with other laboratory duties as needed.

**TECHNICAL OBJECTIVES FOR THIS REPORTING PERIOD**

*Lee*

- The main objective of this period was to assess the viability of anaerobically stored red blood cell's (RBC's) in humans by in vivo 24 hr survival experiments using radioisotopes. Another objective was to begin the second half of the paired in vivo study evaluating the effect of the new additive solution alone.
- A third objective was to continue studying ways to optimize the preservative solution by storage and in vitro experiments.

*Shea*

Alzheimer project:

- To continue the experiments in the NMDA lesioned rats by increasing the number of observations at various time points post lesion.

Oxygen toxicity project:

- A number of rats implanted with microdialysis probes will be run in the new hyperbaric chamber under various depths in order to test the microdialysis equipment under 100% oxygen levels. If all goes well we will then run rats which will receive neurotransmitter releasing stimulation before and during a dive.



Dityrosine Project:

- The compound dityrosine, presumed to be formed from free radicals is not commercially available. Therefore we are attempting to purify this compound. When this is accomplished we will then set up a method for quantitative analysis of dityrosine in microdialysis, CSF, and samples of brain tissue.

*Obowa*

- Complete immunohistochemical staining of tissue sections from bulbectomized rats for detection of c-fos.
- Assist with decompression studies. Refine procedure for NADPH diaphorase staining of spinal cord and brain tissues.

*Porter*

- To continue with fleet soda lime analysis as samples come in from the manufacture.
- To continue the testing program for the hyperbaric CO2 analyzers approved for fleet use.

**SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING PERIOD**

*Lee*

- The first half of the study evaluating oxygen removal was completed. Nine of ten volunteers were able to complete this half of the study yielding the 24 hr survival data. In vitro diagnostics (ATP, hemolysis, vesicle production, lactate, and glucose levels) are done on a weekly basis and will be done for greater than sixteen weeks from the day the blood was initially drawn.
- The second half of the study has begun. Four of eight units are currently in storage while the next four will be drawn in December; infusions begin mid-January and will run through February.
- Repeated experiment to assess varying levels of adenine used in the new blood additive solution. Routine in vitro diagnostics are done weekly to assess the progress of the experiment.



- Began an in vitro experiment to study the levels of lactate in stored RBC's using oxygen removal and the new blood additive solution.

*Shea*

Alzheimer project:

- The number of animals at various time points after NMDA lesioning has been increased.
- The analysis of neurotransmitters is on schedule and has been included in the results.

Oxygen toxicity project:

- Equipment for the diving chamber microdialysis experiments has been completed and is in good working order. We have completed a number of animals in which we elicited neurotransmitter release by perfusing with 100mM potassium for 10 minutes during a baseline period and during a dive at 2 atm. under 100% oxygen exposure. The 20min. samples completed over a six hour experiment were analyzed for dopamine, serotonin, and a number of amino acids.

Dityrosine Project:

- We have received a purified standard preparation of dityrosine from Ajay Verma and with this have been able to develop an electrochemical assay using microbore HPLC for measuring this compound in several biological matrices. We have performed a number of animal experiments primarily using microdialysis to see if pharmacological stressors or environmental factors would cause an increase or decrease in the levels of extra-cellular dityrosine in these animals. Human CSF from various individuals suffering from Alzhiemers' and other mental disorders have also been evaluated for dityrosine levels.

Presentations, Publications, Abstracts, etc.

- Shea P. A., Kerr T.M., Cortes C.M., Ahlers S.T. Nadi N.S., and Auker C.R. (1996) Development of a Microdialysis Procedure to Measure Neurotransmitters During Exposure to Hyperbaric Oxygen. 26th Annual Meeting, Society For Neuroscience.
- Cortes C.M., Shea P.A., Ahlers S.T., Auker C.A., Verma A., Elayan I., and Schrot J. (1996) Measurement of Dityrosine After Exposure to Hyperbaric Oxygen and Chronic Administration of Corticotropin Releasing Factor. 26th Annual Meeting, Society For Neuroscience.



*Obowa*

- Stained brains of bulbectomized and control animals for c-fos immunohistochemistry.
- Stained brain and spinal cord tissues for NADPH diaphorase to demonstrate nitric oxide synthase (NOS) activity in neurons and cerebral microvasculature in animals affected by decompression sickness.
- Performed perfusion fixation of animals and removed brain and spinal cord. Cut brain and spinal cord sections using cryostat. Assisted investigators with dive chamber operation while diving rats for different projects. Ordered lab equipment and supplies.

*Porter*

- A laboratory test plan was formulated for the Dry Deck Shelter hyperbaric CO2 analyzers.
- Six hyperbaric CO2 analyzers completed extensive laboratory development and testing in accordance with the plan developed.
- As a result of laboratory testing the six hyperbaric CO2 analyzers were approved by NAVSEA PMS395 for field testing aboard US Navy submarines.
- Trained US Navy Seal Delivery Teams on proper use of the hyperbaric CO2 analyzers at both east and west coast bases.
- 8 buckets of Military grade soda lime were tested and approved for fleet use.
- Performed other laboratory as requested.

Presentations, Publications, Abstracts, etc.

- Co-authored and constructed a poster presentation "Hyperbaric Carbon Dioxide Analyzer for Dry Deck Shelter Operations" presented at the 1996 Undersea Hyperbaric Medical Society meeting in Anchorage, Alaska.

**GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD**

*Lee*

- Conclude the second half of the paired in vivo study. Evaluate the next step in the investigation of the storage of RBC's and submit protocol changes to the FDA, and UNM Institutional Review Board.
- Continue to establish RBC morphology as an in vitro diagnostic parameter.





- Continue to evaluate lactate levels and its precise effects on RBC's.

*Shea*

Alzheimer project:

- Finish the time course study for post lesioning NMDA animal studies.

Oxygen toxicity project:

- Future work with this project will be to assess the effects of 100% oxygen on the levels of various neurotransmitters in the brain of animals subjected to various depths within the chamber.

Dityrosine Project:

- We are in the process of submitting for publication the new HPLC method for measuring dityrosine. Further improvements to this assay will be continued. The main effort for this method however will be to determine if we can find a biological response to a change in the levels of dityrosine to stress via drug induced or by environmental factors. We will also continue to look for changes or differences in levels of this compound under various human pathological conditions.

*Obowa*

- Nitric oxide synthase studies in progress for decompression may be utilized in the oxygen toxicity research area.
- Planned decompression studies will be focused towards study of the cerebral microvascular components of injury. The role of endothelial cell adhesion molecules and cytokines in ischemia reperfusion injury will be evaluated utilizing histochemistry and immunohistochemical techniques, also counter receptors to these molecules on white blood cells will be studied using flow cytometry. Blood or plasma markers of decompression sickness will be evaluated for usefulness in prediction of decompression outcome in rodents and swine.



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*Porter*

- To continue analysis of fleet soda lime for contaminants and dye concentration as needed.
- To continue testing program for dry deck shelter hyperbaric CO2 analyzers that will be issued to Seal Delivery Teams for fleet use.
- To begin work on new tasking to develop and implement a field test plan for divers air bank sampling on 688 class submarines.
- To begin work on new tasking to develop a CO2 analyzer to be used in a disabled submarine scenario.



GEO-CENTERS, INC.

## **II. NMRI, Bethesda, MD**

### **B. PERSONNEL PERFORMANCE ENHANCEMENT STUDIES**

#### **DESCRIPTION OF WORK TO BE PERFORMED**

##### *Wolf*

- Provide management support to the Naval Medical Research and Development Command. Duties include reviewing medical research plans and progress reports, recommending laboratory guidance, evaluating research proposals, drafting periodic and ad hoc management reports and developing presentation materials.

##### *McCowin*

- Provide management support to the Special Operations Forces Medical Technology Development Program at the Naval Medical Research and Development Command. Duties include reviewing and evaluating medical research proposals, reviewing incremental reports and comparing them with the approved research plans, recommending guidance, and drafting periodic and ad hoc management reports, developing presentation materials and managing financial budget. The scope of research includes all topics within the Special Operations Forces Medical Technology Development program. This includes investigations relevant to the treatment of disease, trauma, effects of environmental extremes and treatment for medical support of Special Operations Forces Operation. In addition, from time to time, collect, process and report findings on critical issues which are directly related to other urgent military medical research issues within the purview of Special Operations Forces Medical Technology Development Program.

#### **TECHNICAL OBJECTIVES FOR THIS REPORTING PERIOD**

##### *Wolf*

- Continue to identify those areas where GEO-CENTERS, INC may assist NMRDC in the transition to whatever its future will be (it should be noted that this remains a difficult task in that we do not know from day to day (sometimes hour to hour) what the future is). Facilitate the production of reports for the Director of Research and Development.



*McCowin*

- Collect monthly obligation and expenditure reports from principal investigators.
- Reviewed and distributed 3rd incremental progress reports.
- Prepare and submit contract package for Duke Univ. to ONR
- Send request for pre proposal to a FY97 New Start Project.
- Send request for proposal package to a project manager of FY97 new start Tasking Statements.
- Submit FY95, FY96, and FY97 obligations and expenditure report and obligation plan to Special Operation Acquisitions Center (SOAC) for Execution Review Conference.
- Submit monthly FY95 and FY96 unobligated funds report to SOAC
- Prepare and submit draft Memorandum of Agreement (MOA) between US Special Operations Command (USSOCOM) and The Naval Medical Research and Development Command (NMRDC).
- Attend Biomedical Initiative Steering Committee (BISC) meeting September 1996.
- Evaluate work unit file of principal investigators for funding and deliverable status.
- Submit final baseline agreements for all SOCOM funded projects to be signed by Program Manager, BISC Chairman, and SOAC Deputy of Acquisition.
- Prepare new baselines for FY97 new start SOCOM funded projects.
- Prepare FY97 funds for distribution in conjunction with BISC chairman and NMRDC comptroller.
- Prepare and submit FY97 funding requirements for Med-Tech program for the Program Objective Memorandum (POM).
- Prepare and present FY98 budget briefing to SOAC Duputy of Acquisition and OSD/OMB.
- Brief Program Manager, Comptroller, and Commanding Officer on the Status of the new Program Base Accounting System (PBAS).
- Attend PBAS training course.
- Install PBAS training and TELNET capabilities on the Comptrollers computer and Program managers computer.
- Provide technical data and input for the revision of the USSOCOM Technology Execution Plan.
- Prepare files of all USSOCOM projects for the incorporation into new 3-D Uninex-based computer program system.



## **SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING PERIOD**

### *Wolf*

- Continued to build a data base for investigator accomplishments. It is the goal of this data base to allow the Director of Research and Development to readily pull together all the significant actions which have been accomplished by our funded investigators. It is intended, also, that this data base will feed into the major work-unit-tracking data base the command has commissioned from a separate contract.
- As a "beta tester" for this command information management system, I have continued to exercise the NavyRIMS system. Working with my colleagues, I've been able to resolve two major and a number of minor performance errors. It is a dynamic process, so this project will continue for some time.
- Reviewed the after-action critiques of the August Wargame "VANGUARD 96", the FIRST medical wargame. Prepared an informal analysis of the critiques and presented same to the Director of Research & Development.
- Final closure was reached and the grant was signed for the Coastal Cancer Research Program at Medical University of South Carolina.
- Prepared two brief packages for the Diving and Submarine Medical Research Area Manager.
- Prepared the NMRDC FY95 Independent Research Report for timely submission to ONR. It must be noted that this year's report was directed to be less formal than in previous years; we were able to complete the package in only one week, and did not put a fancy cover or binder on it.
- Have initiated work to support the Independent Research Manager as she prepares for the ONR directed and sponsored Micro- and Macro-Reviews of the Independent Research Program.

### *McCowin*

- Work from reporting period objective section was performed during this reporting period.



## GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD

### *Wolf*

- Continue to identify those areas where GEO-CENTERS, INC may assist NMRDC in the transition to whatever its future will be. The continuing volatility of this situation makes it difficult on all hands in the planning of such a transition, but the simulations are interesting, that is for certain.
- Continue to support the Independent Research Area Manager and the Diving Medical Research Area Manager as well as the Combat Casualty Care Research Area Manager in the routine functions.

### *McCowin*

- Attend Special Operations medical Association (SOMA) Meeting and USSOCOM BISC Meeting in December 1996.
- Collect and Evaluate 1st incremental progress reports.
- Collect monthly obligation and expenditure reports from principal investigators.
- Submit monthly obligation and expenditure reports to SOAC.
- Prepare and submit final draft of the MOA between US Special Operations Command (USSOCOM) and The Naval Medical Research and Development Command (NMRDC).
- Provide input for the reversion of the USSOCOM Project Reference Book.



## **II. NMRI, Bethesda, MD**

### **E. BREAST CARE CENTER**

#### **DESCRIPTION OF WORK TO BE PERFORMED**

##### *Patient Service Representatives*

*Grimes, Jenkins, Lozoya, Williams*

- Develop a system for processing and interviewing patients, incorporating standard patient registration procedures. Maintain uniform policy for check-in/check-out procedures.
- Devise a system for completion and collection of third party insurance forms on each patient.
- Perfect receiving patients and incoming telephone calls/inquiries, determine priorities and refer to proper person/department.
- Ensure that all incomplete patient records and third party forms are corrected or returned to proper staff for completion/correction.
- Set up records and filing system for paperwork associated with each patient record. Ensure that all documents processed are in accordance with department standards and that all forms are in designated order in the patient records. Label files for permanent shadow files.
- Assist with establishment of standard operating procedures.
- Orient new support team members and clinical team staff to office routine.
- Devise a system of notifying all no-shows, record information in shadow file and initial.
- Print Composite Health Care System (CHCS) daily schedule and end of day reports.
- Check end of day report for accuracy.

##### *Balintona*

- Responsibilities include addressing the psychosocial status, mental status, patient concerns, and the impact of diagnosis on family relationships of breast cancer patients.
- Assess newly diagnosed breast cancer patients and provide them with social work educational materials. The assessment includes a screening for depression, adjustment, patient social history and support systems available to patient.
- Facilitation of the Stage I & Stage II Breast Cancer Survivors' Group



- Facilitation of the Advanced Breast Cancer Support Group on Thursday mornings at 8:30am
- Facilitate the Spouse's of Breast Cancer Patient's Support Group. New men's group to start in January.
- Collect and analyze research data on the Adjustment and Social Support in Male Spouse's of Breast Cancer Patients.
- Liaison with the National Naval Medical Center Social Work Staff. Attend all social work staff meetings to coordinate communication and colleague interaction.
- Coordinate individual, family, group and marital psychotherapy based on Social Work assessment and clinical intervention needed
- Organize and run the Monthly Educational Meetings for patients. Each month features a different topic relevant to patient concerns. The December topic is Hope, Spirituality and Coping.
- Key contact person for the Look Good, Feel Better program run in the Breast Care Center. This program is offered in our center to facilitate coping and increase patients' self-esteem during radiation and chemotherapy treatments. The responsibilities of this program are to keep monthly contact with the American Cancer Society and promote the program to our patients.

*Durand*

- Acts as a liaison between the patient and the family and all other health care providers, intervening at key points (and or when significant problems occurs) for individual patient. Addresses and resolves issues that have a negative impact, creating opportunities and systems to enhance positive outcomes.
- Performs on site visits with patients in various clinical areas.(i.e. Radiology Oncology (Rad. Onc.) Medical Oncology (Med. Onc.) and Post-Op areas.
- Initiates and contributes via multidisciplinary team approach modifications or changes in caregiver practice patterns to maximize quality patient care and resource utilization.
- Assists in the development and implementation of the Care Manager program with Ellora Corporation.
- Checks daily for outstanding biopsy results.
- Page and inform physician of biopsy results if positive.
- Make follow-up phone calls to post operative patients to check on their well-being, this can be weekly, monthly, and every 3 months for maximum of 6 months, then prn.(whenever necessary.)





- Schedule follow-up appointments to Rad. Onc, Med.Onc, Physical Therapy, Nuclear Medicine, and C.T. Scan etc. and various other referrals when ordered.
- Verify consults to clinical areas for breast cancer patients with follow-up phone call.
- Follow-up visit to clinical areas with patients after surgery.
- Verify surgical dates via surgical clinic and OR schedules.
- Perform pre-op teaching specific for nurse case manager with patient and family members.
- Acts as support system for patient and family , in conjunction with the Social Worker and Nurse Educator for newly diagnosed cancer patients.
- Acts as supervisor for 2 Registered Nurses preparing evaluations and preparing time sheets exclusively.
- Instructs post-op mastectomy patients on breast prosthesis, and issues dealing with the appointments for fitting.
- Initiate order forms with prescription for the patient's acquisition of breast prosthesis.
- Acts as liaison between prosthetic company and patient/clinic.
- Hand delivers consults to various clinical areas.

*Richman (Fields)*

- Perform technical services including mammograms.
- Assisting in biopsies and ultrasounds.
- Perform quality control

*Higgins*

- Continuing to define and develop the roles and responsibilities of the Research Nurse position
- Protocol development and a review of the research process
- Discussion and planning of data base development within the BCC
- Complete BRCA education protocol
- Finalize data management of Tam/4-HPR study with NCI
- Utilizing the "Care Manager" to identify trends of care in the BCC
- Keeping the BCC staff abreast of research issues relevant to patient care and staff development
- Attending seminars/conferences for staff and professional development
- Co-investigator on a multidisciplinary team of researchers involved with BRCA1 and BRCA2 testing in the BCC



- Participation and case study presentation at BCC staff meetings and multidisciplinary meetings
- Assisting with eligibility assessment and checklist to register patients into studies
- Ensuring "informed consent"
- Patient education: protocol info, drugs, management of side effects and schedule of care
- Assessment of patient's psycho-social and physical needs
- Providing emotional support to patient and family
- Follow-up and assessment of patients at home
- Maintaining research chart on each patient
- Collection of data - reports of tests, etc.
- Drawing research bloods
- Computerization of data, QA of data
- Preparing agenda and minutes for NCI/NNMC research meetings
- Organizing work area for blood draws and patient consultations
- Work with NCI re: Complementary Medicine and Alternative Therapy

*Lopez*

- Develop and integrate a breast care educational program for female Department of Defense beneficiaries and their support persons.
- Educational program to include all breast care issue with an emphasis on early detection of breast cancer.
- Provide pre-operative teaching and educate patients regarding breast cancer and treatment options.
- Being available as an information resource person for the patient and their support person.
- Plan staff development programs and maintain BCC staff development records.
- Act as relief Ambulatory Care Nurse under the direction of the nurse manager.
- BCC designated safety representative, responsible for safety manuals, monthly safety meetings and BCC safety issues.



*McIntyre*

- Support a research program which focuses on breast cancer.
- Liaison between the Radiology Department-Mammography Section, the Breast Care Center (BCC), and other hospital departments.
- Perform nursing duties.
- Perform managerial duties.

*Portee*

- Coordinates patient flow activities
- Performs professional nursing assessments
- Teaches breast self examination
- Prepares patient charts with appropriate medical, lab, and xray reports
- Assists physicians with all procedures such as FNA or cyst aspirations
- Provides physical and emotional support to patients during their appointment
- Collaborates with a multidisciplinary staff concerning patient needs and identifies patients who may benefit from services such as social service, physical therapy, or nurse case management
- Responsible for preparing all clinical areas for patients and securing clinical areas at the end of the day
- Processes linen and hazardous wastes within the BCC
- Maintains supplies at par level and records supplies needed

*Prindle*

- Coordinates patient flow activities
- Acts as relief clinical nurse manager in the absence of nurse manager
- Collaborates with physicians concerning unscheduled patient appointments
- Performs professional nursing assessments
- Teaches breast self examination and pre and post biopsy education
- Triage patient phone calls and consults with physicians as needed
- Prepares patient charts with appropriate medical, lab, and x-ray reports
- Responsible for entering physician orders into computer
- Assists physicians with all procedures such as FNA or cyst aspirations
- Provides physical and emotional support to patients during their appointment



- Collaborates with a multidisciplinary staff concerning patient needs and identifies patients who may benefit from services such as social service, physical therapy, or nurse case management
- Management of clinical supply needs
- Supervising clinical nursing staff: 1 RN and 1 LPN
- Team Leader for clinical practice

*Snee*

- Case manages new breast cancer patients
- Utilizes the "Care Manager" software to document and track the patient's progress through the clinical care pathway of breast cancer treatment
- Helps to educate newly diagnosed breast cancer patients about disease, treatment, and follow up care
- Provides educational materials to patients and families
- Coordinates and plans appointments for multidisciplinary care in hospital, including, but not limited to hematology/oncology, radiation/oncology, plastic surgery, physical therapy, and social services
- Teaches patients about prosthetics and assists patient in preparing appropriate forms necessary to obtain prosthetic
- Provides emotional support to women and their families who are facing cancer treatment through verbal and nonverbal communication
- Provides support, comfort, and education to the patient through the use of pre and post op phone calls and by visiting the patient while they are an inpatient.
- Ensures that patients are receiving adequate follow up care
- Tracks breast biopsies and notifies doctor of any malignant pathology reports and ensures that patient is scheduled for appointment with physician
- Teaches and demonstrates the "Care Manager" software to interested personnel both within NNMC and at outside facilities
- Assists as needed in clinic as either ambulatory care nurse or nurse educator

*Taylor*

- Manage and maintain the conference room schedule and database.
- Assist with research projects as well as devise a database to store the data.
- Perform desktop publishing, word processing, and other secretarial/clerical functions.
- Provide end user support.
- Write, edit, update correspondence and standard operating procedures



- Maintain electronic filing system.
- Order supplies for various departments within the center.
- Manage and maintain the procurement process and database.
- Generate reports relative to supply issues
- Point of contact for procurement and the conference room
- Assist Patient Service Representatives.
- Attend meetings as assigned.
- Ensure all mail is picked up and delivered daily.
- Organize and label supplies.
- Monitor supplies on hand

*Vaughn*

- Medical filing for the Radiology department and the Breast Care Center.
- Enter CHCS orders for comparison mammograms.
- Track films.
- Handle mail and telephone correspondence regarding radiology films.
- Pull and file mammograms.
- Make copies of mammogram films for physicians.

*Wallace*

- Coordinate administrative activities of the Breast Care Center (BCC). In absence of Administrator and Nurse Manager, act as primary administrator of the BCC.
- Manage workload collection system, ensure reliability of data, collect and report all workload. Monitor accuracy of Care Manager and other data sources.
- Manage budget. Maintain close working relationship with Budget Department, investigate future funding alternatives.
- Monitor legal issues. Make Staff Judge Advocates office aware of potential litigation.
- Work with administrative team to develop Strategic Plan, Information Systems Plan and Marketing Plan for guiding future clinic operations.
- Oversee procurement ordering process. Ensure appropriate supply management system.
- Manage patient/physician schedule templates in the Composite Health Care System (CHCS).
- Oversee use of the Ambulatory Data System (ADS) for the BCC.
- Assist Contract Management Department with maintaining accurate and complete files on BCC employees.



- Assist in preparation for VIP tours and briefings.

### TECHNICAL OBJECTIVES FOR THIS REPORTING PERIOD

#### *Patient Service Representatives*

*Grimes, Jenkins, Lozoya, Williams*

- Streamline and organize front-desk procedures.
- Retrieve and ensure completion of third party insurance forms
- Improve routing and response to incoming telephone calls/inquires
- Use standard registration procedures requiring plastic green card for imprinting all forms pertinent to each patient.
- Coordinate policies for scheduling appointments/procedures for patients calling/walk-ins/consults/cards.
- Streamline physician schedule notification process.
- Refine CHCS daily schedule and end of day reporting.

#### *Balintona*

- Completed and passed the LCSW-C examination in Maryland. This increases opportunity to for BCC to bill patients for psychotherapy provided and increases social workers ability to diagnose/treat patients.
- Collaborated with the NNMC Social Work Department to ensure coverage during my maternity leave this quarter.
- Recruited three additional members for the Advanced (metastatic) Breast Cancer Support Group. The total group number is now seven women. This provides flexibility to the group leaders and increases patients' ability to relate to numerous metastatic women regarding coping, adjustment and psychsocial stressors.
- Beginning involvement with the BRCA Gene Study. Social Worker will serve as individual providing therapy to patients who experience anxiety, depression or other feelings related to the gene testing process and results of the tests. Examining the option of starting a genetic support group.
- Continued to increase Social Work integration with the Breast Care Center Care Manager computer program. This includes formation of a social work critical pathway, patient integration and chart documentation according to the Care Manager format.
- Provided individual psychotherapy to patients experiencing significant emotional distress following diagnosis. Also provided on-going therapy to patients who have



experienced specific types of concerns at the completion of treatment including sexuality/intimacy issues, fear of reoccurrence and family support post treatment.

- Addressed the psychosocial status and mental status of individual patients in the Breast Care Center.

*Durand*

- Continue to improve Care Manager through ongoing collaboration with Ellora Coporation.
- To continue to develop through multidisciplinary collaboration, Standard Operating Procedures (S.O.P.) for the Nurse Case Manager's position.
- Continue to update "Care Manager" data base with retrospective cases of breast cancer patients originally seen in the surgical clinic 1 year ago.
- Continue to enter in "Care Manager" program all Newly diagnosed Breast Cancer Patients/ All new Biopsies.
- Refine techniques for capturing statistical data that would impact future studies of the Breast Care Center.
- Research practice guidelines for Outpatient/Breast Ca. patients, that would apply to BCC.

*Richman (Fields)*

- Perform various studies within the department thereby increasing knowledge and experience.
- Broaden understanding of the BCC's procedures and personnel. Expand relationship with BCC.
- Will take full advantage of any educational opportunities which may arise as time and schedule permits.
- Continue to increase knowledge of mammography and breast diseases using the doctors as teachers.

*Higgins*

- Investigative tool to assess variables for nursing research within the BCC
- Organization of work area and files to begin Research Nurse position
- Further develop computer skills utilizing Windows -NT software
- Working with "Ellora" to utilize the "Care Manager" software as a research tool



- Utilize "Guidelines for Research Proposal" program to start to develop protocols for BCC
- Begin to utilize statistical software programs
- Organize work area and locked cart for needles/syringes for blood draw
- Developed phlebotomy competency checklist for evaluation
- Ordering of supplies needed for venipuncture
- Create data collection checklist and calendars for patient education re: protocols

*Lopez*

- Continue to provide patient education.
- Continue to act as relief ambulatory care nurse.
- Continue to develop array of patient educational materials.
- Continue staff development and safety representative responsibilities.
- Continue to construct SOP manual.
- Prepare for JCAHO survey.

*McIntyre*

- Assist the Radiologists/staff with stereotactic and ultrasound guided breast biopsy procedures.
- Perform assessments on all stereotactic/ultrasound biopsy patients and provide these patients with post breast biopsy teaching instructions.
- Assist with continued development between the BCC and Radiology Department.

*Portee*

- Continues development in the role of the ambulatory care nurse
- Inventory of needles and syringes
- Continues development of computer skills, especially the use of hospital's system called CHCS
- Ongoing evaluation and revision of nursing assessment tool
- Continues to gain further knowledge and education in breast cancer and it's treatment





*Prindle*

- Continues development in the role of the ambulatory care nurse
- Continues development of computer skills, especially the use of the hospital system called CHCS
- Ongoing evaluation and revision of nursing assessment tool
- Continue to gain further knowledge and education in breast cancer and it's treatment
- Ongoing development and assessment of nursing protocols for telephone triage
- Continue working with Patient Service Representatives to achieve a fluid transition between PSR/Patient/Nurse (Developing Algorithm format)
- Develop role as Team Leader for clinical practice
- Participate in staff interviews for clinical nursing positions
- Supervise/Train new staff nurses in clinic procedures
- Schedule new staff into computer training courses
- Develop new appointment template system to better utilize patient flow
- Develop new chart system with management staff to accommodate multiple disciplines : rad/onc and hem/onc
- Develop new algorithm format with Digital for clinical process
- Complete 90 day performance review for LPN position
- Increase nursing continuing education instruction
- Develop new position descriptions for the ambulatory nursing staff

*Snee*

- Ongoing development in the role of the nurse case manager
- Implement processes that will enable appropriate follow up care for breast cancer patients
- Continues to revise and perfect methods to discuss cancer diagnosis with patients
- Continues to gain further knowledge and education in breast cancer and it's treatment
- Ongoing development of organizational skills to manage multiple patients and their individual needs

*Taylor*

- Streamline and organize office procedures to promote a work smarter environment.



*Vaughn*

- Alphabetize the main mammography file system.
- Systematic checking for quality improvement.
- Improve report filing to allow for more efficient operations.
- Being readily available for assistance to CO-workers, the BCC staff, physicians and patients requiring assistance with mammography films.

*Wallace*

- Coordinate administrative activities of the BCC.
- Assist in preparation of Statements of Work and proposals for obligation of future funding. Complete and submit expenditure of funds reports to the Budget Department and Tricare Region 1.
- Manage schedule templates for BCC attending physicians.
- Manage workload collection. Investigate integrity of data.
- Develop system for expenditure tracking, and research patient level accounting system.
- Coordinate research on alternative method of patient charting.
- Develop Strategic Plan and Marketing Plan, Information Systems Plan.
- Oversee use of ADS in the BCC. Ensure compliance with the Surgeon General's standards.
- Attend weekly meetings of the Information Management Quality Management Board to keep up-to-date on all information systems issues.
- Oversee procurement ordering process. Make sure all necessary supplies are ordered in a timely fashion. Ensure proper documentation.
- Provide information to Contract Management to ensure accuracy of their files.
- Participate in genetics research and cancer database development working groups.
- Enhance communication with Hematology Oncology Department to maintain successful working relationship.



## **SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING PERIOD**

### *Patient Service Representatives Grimes, Jenkins, Lozoya, Williams*

- Continued organization of front-desk procedures
- Assisted in development of standard operating procedures.
- Processed and interviewed patients through CHCS and designated forms, obtained and updated all patient demographic information and ensured completion of forms.
- Obtained and verified pertinent insurance information utilizing available forms. Obtained third party insurance forms from physicians at end of each visit.
- Required identification card from each patient and imprinted all clinic forms pertinent to that patient.
- Received patients and incoming telephone calls/inquiries, determined priorities and referred to the proper source.
- Explained clinic procedures to patients.
- Retrieved/returned Mammogram films daily.
- Obtain authorization for release of mammogram films from patient, for NNM file tracking.
- Open monthly clinic schedules and make changes as necessary, based on physician schedule changes.
- Ensured completion of incomplete patient records and third party insurance forms.
- Set up records and maintain filing system for paperwork associated with each patient record. Ensured that all documents processed are in accordance with department standards. Filed all forms in designated order in patient record. Labeled files for permanent shadow files.
- Scheduled and coordinated front desk procedures in accordance with department policy. Identified process problems and helped develop suitable solutions.
- Oriented new support team members and clinical team staff to office routine.
- Participated in team planning to assure team members meet team quality standards. Maintain department standards of productivity.
- Notified physicians the day before they are scheduled for clinic; let them know approximately how many patients they will have.
- Began use of the Ambulatory Data System (ADS), a new computer system for collecting outpatient workload data. Includes ensuring completion of forms and scanning.



*Balintona*

- Addressed the psychosocial status, mental status and patient/family concerns in the Breast Care Center
- Compiled a list of wig salons for patient who are undergoing chemotherapy. This further enhances patient access to services and empowers patient to locate a wig which can increase self-esteem.
- Development of the Social Work Assessment that can be completed on computer. This enhances the social workers efficiency because of time savings and rapid chart documentation.
- Worked closely with the BCC Nurse Case Managers to provide seamless care to patients. This includes daily integration and discussion of services provided to ensure patient care continuity and enhanced patient satisfaction.
- Liaison with the National Naval Medical Center Social Work Department. This included integration with the Social Work department with the signing of a Memo of Understanding signed by the director of Breast Care Center and NNMC Social Work Department during the current reporting period. This ensures that social work coverage will be provided in absence of BCC social worker (during a time of unplanned illness or planned vacation days).

*Durand*

- Have assisted Ellora Corporation in the development of the Care Manager.
- Have worked collaboratively with staff members on other units, and have successfully established a system of making appointments in advance of receipt of consults.
- Have successfully entered approximately 18 new Breast Cancer patients and 42 new diagnostic procedures in the Care Manager system.
- Have resolved critical issues between patient and providers concerning their methods of treatment.
- Have been influential in the initial process of "Outcomes Evaluation " with in the BCC. By contributing a fact sheet on " Outcomes Measures" that included an explanation of 1. Cost/charges of care, 2. Quality 3. Patient Outcomes, 4. Satisfaction with care.
- Continue to demonstrate on an ongoing basis the functions of the "Care Manager" (developed in collaboration with Ellora) to various visitors and dignitaries.
- Continue to generate statical data for monthly evaluation within the Breast Center.



- Was influential in the reassignment of Msgt F. Macdonald from the Mexican Embassy, to Quantico VA. during the surgical and recuperative phase of his wife's breast cancer treatment.

*Richman (Fields)*

- Performed a variety of mammograms, stereotactic biopsies, needle localizations and ultrasound procedures.
- Interfaced with mammography doctors to increase knowledge in the areas of mammography and breast disease.
- Became more familiar with the BCC personnel.
- Obtained CPR certification.
- Received 3 CEU from Eastman Kodak for film demonstration.
- Established QA protocol for the stereotactic biopsy machine.

*Higgins*

- Completed the orientation process of the Research Nurse position
- Enhanced nursing knowledge base on genetic testing and counseling and bio-markers
- Further developed personal computer skills
- Attended seminars/conferences on breast cancer issues and professional nursing issues
- Worked with NCI nurses to develop BRCA educational protocol for patient education
- Participation and case study presentation at BCC conferences
- QA of data within the "Care Manager" and the shadow files of breast cancer patients
- Continued to work with the Technical Assistant to develop slides and flip chart for patient education
- Met with "Ellora" to discuss potential technical needs and resources of the Research Nurse
- Met with Nursing Faculty and General Surgeon to discuss issues of nursing research
- Developed lab order sets for research labs, working with Main Lab personnel
- Worked in Hem/Onc lab to update venipuncture skills and completed competency checklist
- Discussion with NCI staff re: Complementary Medicine and Alternative Therapy
- Organized inventory list for venipuncture supplies
- Preparation of agenda and minutes for research meetings



*Lopez*

- Continued responsibility as the designated safety representative of the BCC.
- Maintained credentialing data base on all Geo-Center employees.
- Planned and instituted staff education calendar and events.
- Functioned as Clinical Educator providing teaching on breast self examination, pre- and post-operative instruction and breast cancer.
- Functioned as relief ambulatory care nurse providing breast self exam teaching, assisting the physicians with physical exams, procedures, and scheduling of diagnostic test when needed.
- Development of departmental SOP and construction of manual.
- Preparation of educational records for JCAHO training.
- Participated in several health fair/wellness programs.

*McIntyre*

- The above technical objectives were met during the current reporting period.
- Assisted with the re-organization of the mammography scheduling process.
- Supervised other mammography personnel.
- Obtained mammography statistical data for FDA purposes.
- Tracked 6 month follow-up patients with outcome analysis via BCC Task Management Tool.
- Correlated mammography and pathology findings via CHCS.

*Portee*

- Coordinated patient flow activities
- Performed professional nursing assessments
- Provided BSE teaching
- Prepared patient charts appropriately with medical, lab, and xray reports
- Assisted physicians with many procedures done in the BCC
- Provided physical and emotional support to patients
- Collaborated with social service, nurse case manager, clinical nurse educator, physical therapist and many physicians to ensure exceptional patient care
- Disposed of linens and hazardous wastes appropriately
- Prepared needle and syringe inventory document
- Read many journal articles about breast cancer and it's treatment



*Prindle*

- Acted as relief nurse manager for the BCC on several occasions
- Collaborated with many physicians concerning unscheduled patient visits
- Performed professional nursing assessments
- Provided BSE and biopsy teaching
- Triageed patient phone calls and made telephone consults to physicians
- Prepared patient charts appropriately with medical, lab, and x-ray reports
- Entered physician orders into the computer
- Assisted physicians with many procedures done in the BCC
- Provided physical and emotional support to patients
- Collaborated with social service, nurse case manager, clinical nurse educator, physical therapist and many physicians to ensure exceptional patient care
- Developed new role as Team Leader
- Managed/Supervised new nursing staff for the ambulatory care area
- Developed BCC medical reference library list
- Completed algorithm format for clinical process
- Attended Annual Maryland Nurses Association Conference 10/96
- Attended conference on "Ethical Dilemmas and Managed Care 10/96
- Completed 90-day performance review for LPN staff member

*Snee*

- Suggested and implemented useful changes in the care manager software
- Helped to educate patients and families on breast cancer
- Provided emotional support to women from diagnosis to completion of breast cancer treatment
- Collaborated with multiple disciplines to arrange for patient care
- Developed useful methods for managing many varied and complex patients
- Taught many new cancer patients about breast and wig prosthetics and assisted them in obtaining the prosthetics
- Attended tumor board meetings and was prepared to give additional information concerning breast cancer patients if required or requested by physicians
- Attended case management conference and gained very valuable information and ideas that will be beneficial
- Collaborated with staff on the development of a cancer database
- Provided education and working demonstration of the "Care Manager" software to interested personnel both within NNMC and to outside facilities



*Taylor*

- Assisted Clinical Education with writing and reviewing standard operation procedure for the center.
- Assisted Patient Service Representative as well as nurses when/where needed.
- Provided end user support for department staff.
- Created presentation slides for BRCA gene testing.
- Performed word processing for the center's staff.
- Organized supplies to allocate more usable space.
- Ensured a smooth correspondence flow/distribution.
- Maintained 4 databases to assist with streamlining and improving the procurement process, conference room scheduling, and breast cancer research.
- Learned new software to support research projects as needed.
- Maintain the electronic filing system.
- Wrote patient correspondence for appointment and medical issues.
- Generated supply reports.
- Performed upgrades, installs, and troubleshooting for BCC Computer Systems.
- Became Assistant Administrator for BCC computer systems.

*Vaughn*

- Provided assistance to staff requesting help with mammography films.
- Organized log book to improve film tracking.
- Devised a new way to disseminate films to patients via TICS computer input.
- Designed release form for patients signing out mammogram films.

*Wallace*

- Coordinated administrative activities of the BCC. Acted as administrator during the absence of the Administrator and Nurse Manager.
- Collected and analyzed data on open appointments to determine 1) percentage of open appointments daily and 2) need or lack of need for more appointments.
- Addressed legal concern with Staff Judge Advocate's office.
- Worked with nurses to revise and implement filing guidelines for patient charts.
- Supervised research of alternative method for patient charting.
- Researched reliability of Care Manager data.
- Worked on Standard Operating Procedures Manual.





- Assessed Information System needs, including Hardware and Software, for immediate and future needs.
- Managed schedule templates for BCC attending physicians in five subclinics.
- Maintained relationship with Contract Management Department.
- Developed system for budget management expenditure tracking. Developed relationship with new Budget Department Liaison.
- Expanded use of CHCS standard reports.
- Revised budget based on new funding, coordinating with Budget Department to match records.
- Cross referenced BCC supply records with Budget Department supply records.

### **GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD**

#### *Patient Service Representatives*

*Grimes, Jenkins, Lozoya, Williams*

- Become proficient in the use of ADS.
- In effort to continuously improve quality, streamline and organize front-desk procedures.
- Coordinate scheduling with other clinics for smoother follow-up visit for the patient.
- Maintain department standards.
- Attend classes involving CHCS training as well as computer training.

#### *Balintona*

- Expand the BCC social work library to include more texts that address the emotional issues related to breast cancer. (Pt frequently request reading material or suggest books that helped them cope with treatment)
- Begin the next Stage I/II group on 10 JAN 96.
- Continue working relationship with the American Cancer Society to bring programs that address psychosocial issues related to cancer to the Breast Care Center.
- Enhance Care Manager (computer program utilized in BCC for patient care) usage for patient care integration. This objective will include daily documentation on the Care Manager and continued discussion with computer programmers to make it compatible to social work service provision.
- Liaison with Dr. Ken Miller, Nursing Researcher, for further feedback and input on the research proposal.



- Provide social work services to patients by addressing psychosocial status, mental status, patient and family concerns.
- Coordinate individual, family and group psychotherapy for patients.

*Durand*

- Continue to work in collaboration with Ellora to improve the "Care Manager"
- Continue to develop the Standards of Procedures for the position of the Nurse Case Manager.
- Research and develop practice guidelines for the Case Manager in the Breast Cancer setting..
- Attempt to attend at least one conference for Nurse Case Management within the next evaluation period.

*Richman (Fields)*

- Obtain mammography certification.
- Attend an educational mammography seminar.
- Broaden my knowledge of breast diseases and mammography.

*Higgins*

- Continue to enhance nursing knowledge base on genetics, bio-markers and tissue banking
- Continue to further develop personal computer skills
- Continue to attend seminars/conferences on breast cancer issues and professional nursing issues
- Continue to define and develop the roles and responsibilities of the Research Nurse position
- To initiate nursing and clinical protocol development in the BCC
- Utilization of the Care Manager to monitor trends in the BCC and collect research data
- Observe the cancer genetics nurses and learn the counseling process related to genetics
- To fully understand the implications and process of tissue banking
- Provide in-service to BCC staff regarding on-going research



*Lopez*

- Continue responsibility as safety representative
- Continue to function as Clinical Nurse Educator providing teaching to patients and their support persons.
- Continue to function as relief Ambulatory Care Nurse.
- Participate in wellness programs.
- Identify needed materials and supplies for procurement.
- Complete SOP manual.
- Participate in JCAHO survey if completed.

*McIntyre*

- Continue to perform nursing and managerial duties, as described above.
- Continue to obtain mammography statistical data for FDA purposes on a monthly basis.
- Track 6 month follow-up patients with outcome analysis via BCC Task Management Tool.
- Attend nursing/management conferences when available.

*Portee*

- Will continue to enhance education in breast cancer and it's treatment
- Will continue to improve patient flow management
- Will continue to improve computer skills
- Will attend a seminar/conference related to breast cancer
- Will continue to participate in multidisciplinary meetings
- Will continue to learn teaching including biopsy and APU

*Prindle*

- Will develop breast care center medical reference library
- Will continue to enhance education in breast cancer and its treatment
- Will continue development of nursing protocols and quality assurance documents, especially in the area of telephone triage
- Will continue to develop Team Leader Role and patient flow activities
- Will continue to improve computer skills
- Will attend a seminar/conference related to breast cancer.



- Will continue to participate in multidisciplinary meetings
- Will improve upon and fine tune presentation on ambulatory care nursing for future presentations
- Will improve expertise in patient education for APU patients having breast surgery
- Will attend a seminar for Health Professionals in utilizing the Internet
- Will continue to develop new processes to better enhance clinic management
- Complete new nursing position descriptions for the ambulatory clinic staff
- Complete 90-day performance review for RN staff member
- Attend a conference on "Cancer Awareness" in Hershey Pa

*Snee*

- Will develop a workgroup to design the clinical care pathway for breast cancer that relates to the "Care Manager" tool
- Will develop and prepare speech on the case management of the breast cancer patient for international breast cancer conference in March '97
- Will continue to improve skills as a nurse case manager
- Will assist in the development of a breast cancer database
- Will develop concise methods to manage multiple patients
- Will continue to enhance education in breast cancer and it's treatment
- Will continue to improve computer skills
- Will attend a seminar/conference related to breast cancer
- Will continue to participate in multidisciplinary meeting
- Will establish guidelines for case management follow up after the acute stage of diagnosis and treatment of the breast cancer patient

*Taylor*

- Continue with streamlining office procedures and processes to improve efficiency.
- Maintain, improve, and/or introduce databases to improve productivity and organization of data.
- Continue efforts to learn more in regards to computer systems.
- Define a personal development program to assure continuing professional growth.



*Vaughn*

- Continue with duties as described above.
- Reduce turn around time for mammogram films returned to the department from the BCC, General Surgery Clinic and patients.
- Purge duplicate mammogram folders.

*Wallace*

- Continue to nurture relationship with the Radiation Oncology Clinic and the Medical Oncology Clinic.
- Continue participation on genetics research and cancer database working groups.
- Research possibility of ad hoc reporting in CHCS.
- Continue to coordinate administrative activities of the BCC.
- Complete research on alternative methods for patient charting to reduce amount of paper and select a system.
- Continue development of Strategic Plan, Information Systems Plan, and Marketing Plan.
- Increase budget management, actively seek new avenues of funding.
- Monitor legal issues.
- Monitor compliance between BCC supply records and Budget Department supply records.



### **III. NDRI, Great Lakes, IL and NDRI Detachment, Bethesda, MD**

#### **A. DENTAL DISEASES-RELATED RESEARCH**

##### **DESCRIPTION OF WORK TO BE PERFORMED**

###### *Beck*

- Provide technical assistant with ongoing Immunology research projects. Participate in linkage analysis project with Molecular and Epidemiology of NIH. Maintain and upgrade the laboratory such that the research experiments are carried out smoothly. Maintain and record proper technical procedures and data produced for each experiment.

###### *Jones*

- Senior Research Scientist
- Responsible for the Molecular Biological and Molecular Genetic aspects of the projects. This includes the development, evaluation and refinement of molecular biological research protocols.

###### *Turner*

- To work as a Senior Scientist in the Basic Sciences Department of the Naval Dental Research Institute, and as the Principal Investigator on 61152N project "Antimicrobial activities of polymorphonuclear granule components in human periodontal diseases". This project had been approved for funding from 1 October 1993 to 30 September 1996. The principal investigator heads a research team consisting of Mr. Ernest Pederson, HM2 Carolyn Merritt and HM3 Colin Glynn.

###### *Lamberts*

- To assist as an editorial consultant in the preparation or review of manuscripts to be submitted for publication.
- To aid in the preparation of research presentations (such as posters) for scientific meetings, in the review of research proposals, research communications (letters, rebuttals), etc.



*Miller*

- Senior Research Scientist and Group Supervisor. Responsible for all aspects of Immunological, Microbiological, and Tumor Biomarker activities within the Naval Dental School. This includes the development and supervision of research protocols, dental resident mentoring activities, instruction of courses in dental microbiology and dental immunology, serving as a link between NIH sponsored research and Naval Dental Research programs, and troubleshooting of research programs, computers, instrumentation and equipment.

**TECHNICAL OBJECTIVE FOR THIS REPORTING PERIOD**

*Beck*

- Begin experiments on gathered lymphocyte samples by isolating RNA's and subsequently converting to cDNA's and evaluating the PCR products via gel electrophoresis (DNA sequencer).
- Grow and maintain various fibroblast cell lines
- Isolate RNA from the stimulated and non-stimulated fibroblast cell lines
- Assist Molecular and Epidemiology group of NIH with Cleft-Lip Palate (CLP) disorder linkage analysis study

*Jones*

- Relative to the program entitled "Biomarkers for Oral Cancer," work will proceed on the development of appropriate methods for the identification of polymorphisms in genes resulting in increased cancer risk. DNA and/or tissue samples from the oral cancer study and will begin processing them immediately.
- Effort is being focused toward the development of a comprehensive database for the maintenance of data generated in the Biomarkers Study. We will be working closely with individuals from NIH expert in database design and maintenance.
- Will become involved in the investigation of bacterial stimulator-mediated induction of expression of IL-1 and IL-6 specific mRNAs. Will explore the development of an internal standard molecule to facilitate the quantitation of specific cytokine gene expression.



*Lamberts*

- Work on the preparation of the manuscript by E. Pederson et al. on "Heavy Metal Pollutant Removal from Dental Operatory Waste Water"
- Revise the manuscript of S. Schade et al. on "Mitogenic Activity in the outer Membrane of *Treponema denticola*".
- Revise the format of the manuscript of E. Pederson on "Reducing medium for the cultivation of anaerobic *Porphyromonas gingivalis* under aerobic conditions". This paper was not accepted in a recent submission for publication, so will be resubmitted to another journal.
- To assist Naval Dental Research Institute (NDRI) investigators editorially whenever requested.

*Turner*

- To repeat and verify procedures for testing granule components against *Treponema denticola*.
- To continue to fractionate primary and secondary neutrophil granules by Fast Protein Liquid Chromatography and High Performance Liquid Chromatography.

*Miller*

- Relative to the project "Protein Pattern Recognition for risk Assessment of Periodontitis" Navy R and D IR funding is being awaited. Should funding not be forthcoming the project will be halted.
- Relative to the program entitled "Evaluation of disproportionate expression of T-cell receptor V $\beta$  regions in lymphocytes from patients with advanced periodontitis", mRNA's from remaining cultured peripheral blood mononuclear cell reparations will be transcribed to cDNA and the cDNA's subjected to analysis for TCR V $\beta$  chain expression by PCR. It is expected that the majority of this project will be completed in the next quarter.
- Relative to the project "Cytokine production by polymorphonuclear leukocytes resident in periradicular and periodontal lesions a final manuscript is nearing completion and will be submitted for publication.
- Relative to the Biomarkers for Oral Cancer program, work will continue relative to the identification of human papilloma virus DNA in premalignant oral lesions. Efforts will also be directed toward the establishment of a PCR/OLA procedure for identification of specific alleles in various human DNA samples.





- Work will continue on a project designed to evaluate antibacterial activity of currently used endodontal medicaments. It is anticipated that Vitapex® and Calasept® will be evaluated against anaerobic organisms such as *P. intermedia*, *P. gingivalis*, and *A. viscosus* during the next quarter.
- Work will continue toward the quantitation of IL-1 and IL-6 specific mRNA from oral fibroblasts cultured in the presence of various oral bacterial stimulators. In addition, a collaborative effort between our laboratory and that of Dr. M. M. Sholley, Department of Anatomy, Medical College of Virginia will be initiated to look at the influence of bacterial stimulators on cytokine production by endothelial cells in culture.
- Begin teaching Oral Microbiology for Naval Dental Residents.

#### **SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING PERIOD**

##### *Beck*

- RNA's were isolated from frozen lymphocytes and reverse transcribed to make cDNA's. Then, sequences of cDNA's (specific for loci of interest) were amplified and tagged with fluoroscenated markers using polymerase chain reaction (PCR) technique. These PCR products were visualized by running PCR products through acrylimide gels on gel sequencers. Furthermore, gels were analyzed using various software such as genotyper and genescan analysis.
- Several lines of fibroblast are being maintained for continuous growth. These cells, gingival and OT-1 (tumor cell line), were subjected to various growth factors and bacterial cell components to observe modifications at nuclear level. RNA's were isolated from these cells after two days of incubations and stored for future reverse transcription and PCR experiments.
- Fibroblast subjected to the antigens such as bacterial components and growth factors were gathered for RNA isolation and PCR studies. RNA isolation was done on each stimulator groups and stored for future experiments of PCR analysis.
- Continued to participate in NIH Molecular and Epidemiology experiments. This study deals with the inherited genetic disorder, CLP. Primarily, linkage analysis is done by amplifying DNA loci that may be associated with this inheritable disorder via PCR and running gels to visualize the amplified gene sequences.



*Jones*

- Relative to the program entitled "Biomarkers for Oral Cancer," DNA and cells from peripheral blood derived from a case-control study of oral cancer in Taiwan has recently been obtained. DNA is presently being isolated from the peripheral blood buffy coat layer. These DNAs are being screened for several genetic polymorphisms associated with increased cancer risk using PCR, allele-specific PCR and a combination of PCR and restriction endonuclease digestion. In addition, we will continue to identify new genes for analysis.
- Optimization of techniques to identify single basepair alterations in tumor-derived DNA were completed. "Cold" single-strand conformation polymorphism (SSCP) analyses have been carried out on DNA obtained from a small sample of oral tumors with very promising results. This technique will be used in the analysis of specific genetic alterations as soon as DNA and/or tissue samples from tumors arrives.
- With regard to the development of a database for the maintenance of data generated in this study. A comprehensive list of "fields" for inclusion in the database design has been compiled. In addition, preliminary discussions have occurred with individuals from the NIH expert in database design and maintenance regarding the selection of appropriate database programs in order to maintain compatibility with existing systems.
- Relative to the project on cytokine production by oral fibroblasts, specific primers for the analysis of expression of IL-1, IL-6 and IL-8, in addition to primers for the "housekeeping" genes  $\beta$ -action and cyclophilin, have been synthesized. The PCR conditions for the amplification of specific cDNAs derived from various gingival and pulpal fibroblasts cultured with bacterial stimulators, were optimized. Primers for additional cytokines have been identified and the sequences submitted for synthesis.

*Lamberts*

- Completed work on the manuscript of E. Pederson et al. on "Heavy Metal Pollutant Removal from Dental Operatory Waste Water", and on the manuscript of S. Schade et al.
- Completed format revision of the manuscript of E. Pederson et al., retitled "Reducing Medium for the Cultivation of *Porphyrromonas gingivalis*".
- Reviewed 6 abstracts (Pederson, Simonson, Ralls, Schade, Schultz, Simicek) of research studies that have been submitted for presentation at the March 1997 meeting of the International Association for Dental Research.



- Reviewed 3 research posters (Pederson, Halverson, Simicek) that will be presented at several research meetings.

*Turner*

- The effectiveness of Cathepsin G against *Treponema denticola* was verified, along with the lesser microbicidal activity of lysozyme..
- Most of the work has focused on refining the separation procedures necessary to isolate bacterial products that inhibit the microbicidal activity of Cathepsin G against *Treponema denticola*.
- A manuscript reporting details of this work is in preparation.

*Miller*

- Relative to the project "Protein Pattern Recognition for Risk Assessment of Periodontitis" no additional work will commence until funding is available. Preliminary notification was received indicating that our most recent IR proposal (document attached) received favorable comment and that funds will be forthcoming.
- Relative to the program entitled "Evaluation of disproportionate expression of T-cell receptor V $\beta$  regions in lymphocytes from patients with advanced periodontitis" (Work Unit: 0601152N.MR00001.001-0063), all RNA isolations and cDNA synthesis for control and diseased subjects have been completed. cDNA's have been titrated to equalize C $\alpha$  PCR products using the ABI 272 sequencer and PCR work has been completed for all subjects. Gel runs on the ABI 272 have also been completed. An abstract has been submitted for presentation at the International Association for Dental Research Annual meeting in Orlando, FL. In March, 1997.
- Relative to the project "Cytokine production by polymorphonuclear leukocytes resident in periradicular and periodontal lesions" (Work Unit: 0601152N.MR00001.001-0063); a final manuscript has been completed and submitted to the Journal of Endodontics for consideration for publication.
- Work Unit: 0601152N.MR00001.001-0063. Long term frozen storage of lymphocytes. One manuscript entitled "Studies of Proliferative Responses by Long-Term-Cryopreserved Peripheral Blood Mononuclear Cells to Bacterial Components Associated with Periodontitis" has been published in Clinical and Diagnostic Laboratory Immunology. A second manuscript entitled "Cytokine Production by Cryopreserved Peripheral Blood Mononuclear Cells" is nearing completion and will shortly be submitted for publication.



- Relative to the program entitled "Biomarkers for Oral Cancer," a dot blot procedure has been developed allowing an additional way to detect PCR products. In addition, 230 paraffin-embedded oral lesions from 144 patients were re-evaluated in both dot-blot and PAGE using HPV L1 and E6 consensus primers and HPV 16 and 18 type-specific primers. An abstract concerning derived from this work has been submitted for presentation at the International Association for Cancer Research in San Diego, CA. in April.
- Relative to the project Evaluation of the Influence of Superantigens and Polyclonal B-cell Activators in Periodontal Disease (Work Unit 0601152N.MR00001.001-0063) one manuscript reporting these results entitled "Studies of immunomodulatory and superantigen activities of bacteria associated with adult periodontitis has published in *Periodontology*.
- Relative to studies evaluating antimicrobial activity of dental materials which was completed about one year ago a manuscript titled "Antimicrobial activity of dentin bonding systems and glass ionomers" has now been published by the *Journal of Operative Dentistry*.
- Relative to studies designed to evaluate antibacterial activity of currently used endodontal medicaments, Vitapex® and Calasept® and a variety of controls were additionally evaluated against *P. intermedius*, *P. gingivalis*, *A. viscosus*, and *V. parvula*. This work has also been presented at the George Washington University Research Symposia.
- A project has recently been initiated to study cytokine production by oral fibroblasts. This study extends a previous study designed to look at the influence of various growth factor on wound healing. Various gingival and pulpal fibroblasts have been obtained and cultured with bacterial stimulators. Total RNA has been isolated and used to prepare cDNAs by reverse transcription. In addition, primers have been synthesized for use in a PCR based semiquantitative analysis of mRNAs for IL-1, IL-6, and IL-8. In addition, primers specific for the housekeeping genes for cyclophylin and  $\beta$ -actin have also been synthesized. PCR conditions for all of the primers have been optimized.
- The course Oral Microbiology for Dental Residents has been successfully completed.

Presentations, Publications, Abstracts, etc.

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- Cummings, G., Lenoci, J.L., Malick, N.S., D'Alesandro, M.M. and G.A. Miller. Antibacterial effectiveness of temporary endodontic filling materials. 3rd Annual George Washington University Health Sciences research day.
- In collaboration with Dr. M.M. D'Alesandro, MSC, USN "Protein pattern recognition for risk assessment of periodontal disease" for consideration for IR funding.

#### GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD

*Beck*

- Main objective of this upcoming quarter is to continue with NIH-CLP project
- Continue gathering necessary samples for the RNA analysis
- Continue isolating RNA's and convert to cDNA and identify interested locus via PCR



*Jones*

- Relative to the program entitled "Biomarkers for Oral Cancer," it is anticipated that the characterization of a major portion of the genetic polymorphisms in the Taiwan oral cancer study will be completed. The data will be analyzed for differences in the frequencies of these polymorphisms between the cancer cases and unrelated controls to determine if one or more is associated with increased oral cancer risk.
- Arrival of DNAs and/or tissues from a Taiwan-based study of nasopharyngeal carcinoma (NPC) using multiplex families is anticipated. These DNAs will be analyzed for the frequencies of several genetic polymorphisms that have been associated with increased risk of cancer. In addition, these DNAs are to be characterized relative to polymorphisms at the complex HLA locus.
- Effort will continued to be directed toward the development of a comprehensive database for the maintenance of data generated in the Biomarkers Study.
- Continued involvement in the project on cytokine production by oral fibroblasts is anticipated. Methods presently in use are sufficient to permit a semiquantitative assessment of levels of expression of the cytokine encoding genes. Will continue to explore the development of an internal standard molecule to facilitate more precise quantitation of specific cytokine gene expression.

*Turner*

- My submission of a proposal to continue this work during the next fiscal year was not approved, apparently because of a lack of research funds. Hence, this report will represent the completion of my work at NDRI and my employment by GeoCenters.

*Lamberts*

- Completion of work.

*Miller*

- Relative to project "Cytokine production by polymorphonuclear leukocytes resident in periradicular and periodontal lesions" (Work Unit: 0601152N.MR00001.001-0063); work will begin on the development of *in situ* hybridization and *in situ* PCR methodology to permit sensitive identification of cytokine mRNA in tissue sections.



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- Relative to the development of a 2-D electrophoresis procedure to study components of gingival crevicular fluid from individuals with severe periodontitis, work will be re-initiated should IR funds be released.
- Relative to the program entitled "Evaluation of disproportionate expression of T-cell receptor V $\beta$  regions in lymphocytes from patients with advanced periodontitis" (Work Unit: 0601152N.MR00001.001-0063). It is anticipated that this work will be presented at the International Association for Dental Research Annual meeting in Orlando, FL, March 1997.
- Relative to the program entitled "Biomarkers for Oral Cancer," it is anticipated that this work will be presented at the International Association for Cancer Research Meeting in San Diego, Ca. in April and that work will be extended to include a variety of additional tissue biopsies.
- Work Unit: 0601152N.MR00001.001-0063. Long term frozen storage of lymphocytes. A manuscript covering work on cytokine production by long term frozen stored cells will be completed and submitted for publication. This project is not closed.
- Complete teaching the course Oral Immunology for Dental Residents.



GEO-CENTERS, INC.

**V. NMRI TOX/DET Dayton, OH**

**A. TOXICOLOGICAL STUDIES**

**DESCRIPTION OF WORK TO BE PERFORMED**

*Abbas*

- Member of the Pharmacokinetic group.
- Project Director for the largest project (Trichloroethylene Biologically Based Health Risk Modeling) within Tri-Service Toxicology Consortium. The project is funded by Strategic Environmental Research and Development Program (SERDP) \$900K per year.
- Responsible for meeting customer needs, interact with high-level federal government scientists, brief and provide monthly and quarterly status reports to the Science Advisory Board and Executive Chair of the Strategic Environmental Research and Development Program (SERDP).
- Collaborate with Scientists from United States Environmental Protection Agency (USEPA), Wayne State University, Creighton University, University of Wurzburg (Germany), and Zeneca (United Kingdom) to investigate Human toxicity of TCE.
- Lead multiple research teams to development methods, explore pharmacokinetics, pharmacodynamics, metabolism, metabolic pathways of trichloroethylene (TCE) and its metabolites, chloral hydrate (CH), trichloroethanol (TCOH), trichloroethanol glucuronide (TCOG), trichloroacetic acid (TCA) and dichloroacetic acid (DCA).
- Develop a comprehensive Biologically Based pharmacokinetic model for TCE and five metabolites.

*Ademujohn*

- The purpose of the neurobehavioral laboratory coordinator at NMRI/TD is to provide technical support to various aspects of ongoing on-site projects in neurobehavioral research. During this quarter the coordinator has been and will be involved in neurobehavioral testing for the effects of simulated stress factors relating to the Gulf War Syndrome on animal models via computer-aided qualitative and quantitative methods. The coordinator also supervises animal training protocols for upcoming pharmaceutical exposure studies.





*Briggs*

- General Manager and Senior Contractor Representative for the NMRI contract activities at the Toxicology Detachment. Serves as a toxicologist and performs research as an Associate Toxicologist. Responsible for collaborating the GEO-CENTERS, INC.. resources in support of the toxicology research projects in support of the Toxicology Detachment mission.

*Carraci*

- Group Supervisor I/Scientist II
- Member of the Pharmacokinetics Group.
- Study Director for project, Physiologically Based Pharmacokinetic Modeling of Cardiac Sensitizing Chemicals in Rats.
- Perform inhalation experiments for the Total Petroleum Hydrocarbon (TPH) Project

*Connolly*

- Cataloging print and non-print materials for circulation
- Ordering and maintaining serials collection, including claiming missing issues
- Handling reference questions
- Providing interlibrary loan assistance
- Locating needed materials in other libraries
- Preparing book orders

*Garrett*

- Scientist II
- Member of Pharmacokinetics Group
- Member of Dermal Penetration Project Team
- Room Monitor

*Geiss*

- Group Supervisor II/Scientist III.
- Group Administrator for the Pharmacodynamics (PD) Technical Area Group, Tri-Service Toxicology Consortium.
- Methods and protocols development for the molecular biology laboratory.



- Ensure PD support is provided to project leaders.
- Perform ongoing molecular biology analysis for project experiments.
- Participate in project planning.

*Grabau*

- Senior Scientist I.
- Provide scientific image processing and analysis to multiple ongoing research efforts.
- Member of Pathology Technical Area Group.
- Member of Dermal Penetration Project Team.
- Member Trichloroethylene Project Team.
- Member of TriService Marketing/Program Development Team.

*Horton, Rix*

- Maintain LAN
- Maintain and upgrade individual Desktop and Laboratory Computers
- Provide answers, support and expertise in correcting computer problems, including all peripherals attached to these systems
- Continue comprehensive program for maintaining system integrated and reliability through back-up procedures, documentation, and redundant systems
- Continue to update information Databases HMIS, IRIS, Medline and Toxline
- Maintain and Update NMRI/TD Web page and Internet/Intranet services
- Organize Media, Manuals and Spare Parts
- Prepare ASDPs for procurement of new computer systems, software and peripherals
- Develop in-house software and databases

*Jung, Narayanan, T.K.*

Trimethylolpropane (TMPP) Evaluation:

- Measurement of the amounts of neurotransmitters present in brain homogenates using an HPLC method
- Continue culturing the mouse neuroblastoma cell line N2-A
- Analyzed rat and chicken brain samples (provided by CDR Rossi) for their neurotransmitter content

Cell Model Project:

- This project is on hold until further funding is received



*Kimmel, Reboulet, Whitehead*

- Installed fume hood with ducting and modified physical plant (electrical, HVAC, water, sewer, compressed gases - etc) in laboratory room 114, Bldg 824 WPAFB in preparation for installation of an inhalation toxicology/pulmonary physiology laboratory.
- Moved laboratory equipment, inhalation exposure systems, and analytical instrumentation from room 120, bldg 433 to rm 114, bldg 824.
- Co-authored (in collaboration with investigators from Naval Air Warfare Center and the University of Pennsylvania) a proposal (program level effort - 10 years) for the development of a mathematical model of aerosol deposition and retention in the lung, based on physiological, anatomical, aerosol dynamic behavior first principles.
- Wrote three research pre-proposals (inhalation/pulmonary toxicology) for submission to Office of Naval Research.
- Developed draft and preliminary design and concept engineering plan for installation of a new inhalation exposure facility in bldg 839 to dedicated to Neurobehavioral toxicology.
- Started initial stages of Adult Respiratory Distress Syndrome (ARDS) research project.

*McDougal*

- Continue pharmacokinetic studies in hairless guinea pigs for chloropentafluorobenzene and 1,2-dichlorobenzene.
- Emphasize *in vitro* diffusion cells in the development of the mathematical models for the whole animal experiments.
- Complete organ weight data on Hartley guinea pigs and Fischer 344 rats for physiologically based model.
- Complete development of analytical methods for MACs *in vitro* exposures.

*Narayanan*

- Scientist II/Member of the Hazard Assessment Group
- Support combustion and in-vitro toxicology research
- Support studies involving toxicity evaluation of explosives and propellants
- Support projects involving toxicity evaluation of vapor phase lubricants



- Assess neurotoxicity in simulated Persian Gulf War (PGW) exposure in Sprague-Dawley rats

*Prues, Smith*

- Conduct blood gas studies with regard to homeostasis effects following serial blood collection/transfusions.
- Complete data books for SFE range-finding/multiple-dose, edema and blood gas studies.
- Submit revision back to journal editor for final consideration publication on SFE Formulation A pilot studies: part I and Part II.
- Conduct edema studies on SFE Formulation B, C and D.
- Determine the mode of action of TMPP.

*Ritchie*

- Dr. Ritchie serves as Assistant Group Leader for the Neurobehavioral Toxicology Group at the Tri-Service Toxicology Consortium and NMRI/TD and as Associate Principal Investigator (API) for all currently funded neurobehavioral toxicology-related work units (FY97 funding of \$650K).
- Assists in program management, budgetary control and procurement, research design, protocol preparation, research supervision, statistical analysis and preparation of scientific papers and abstracts in five areas of neurobehavioral toxicology research.

TMPP MECHANISMS OF ACTION: DEVELOPMENT OF NEUROBEHAVIORAL MOLECULARIZATION TECHNIQUES (WU .1516):

- Anatomical disposition and neurobehavioral effects of trimethylolpropane phosphate (TMPP), a potent neurotoxicant produced through the pyrolysis of synthetic lubricants used in military ships and aircraft.

Mechanisms Involved with Exposure to Select Neurotoxicants (WU .1712):

- Development, testing and validation of new physiological and mathematical modeling techniques for estimation of expected concentrations of selected toxicants in major CNS regions following dermal, oral or respiratory exposures.



Development of the Navy Neuro-Molecular Assessment System (the NTAS) [WU .1713]:

- Development and validation of a number of neuro-molecular (cellular-level) analytical techniques for eventual inclusion in the NTAS.

Neurobehavioral Toxicity Assessment Battery (NTAB):

- Assessing Animal Responses to Pharmacological Challenge (WU .1605): Predictive validation of the NTAB by comparison of animal and (known) human responses to identical pharmacological challenges on neurobehavioral tests with topographical similarity.

PERSIAN GULF WAR (PGW) SIMULATION USING SPRAGUE-DAWLEY RATS (US ARMY AND NMRI/TD):

- Development of an animal model to simulate exposure encountered by Persian Gulf War veterans.

**TECHNICAL OBJECTIVES FOR THIS REPORTING PERIOD**

*Abbas*

- Prepare a Protocol/Addendum to investigate the kidney tumor related glutathione pathway of TCE metabolism in rats.
- Continue data analysis and determination of metabolic rate constants for TCE metabolites.
- Perform sensitivity analysis on the PBPK model.
- Determine inter-conversion and percent yields of CH, TCOH, TCOG, TCA and DCA.
- Continue and complete the model development.
- Conduct more intensive literature review on TCE, its toxicity, pharmacokinetics, metabolism, and its metabolite toxicity and pharmacokinetics.
- Initiate and prepare a peer review PBPK model paper in mice for *Journal of Toxicology and Applied Pharmacology*.
- Continue to manage the TCE project, meet the customer needs, coordinate and oversee the TCE research work.



*Ademujohn*

The major technical objectives for this period are as follows:

- Testing various pharmaceuticals on animal models using diminished capacity as the endpoint in Carneaux pigeons and Wistar rats.
- Range finding using operant-trained animals and measuring subsequent stages of diminished capacity.
- To compile, catalog and computerize the above mentioned data.
- To train pigeons and rats for problem solving protocols
- Daily maintenance of pigeon intake and logging performance results.
- To obtain operant testing and training data for animals used in operant exposure testing .
- To organize, catalog and generate computer graphics, cumulatively from the above mentioned data.
- To maintain data for future reference in upcoming publications.
- To be responsible for the procurement and securing of all materials used in testing and training protocols.
- Responsible for documenting and maintaining operant weights.
- Responsible for writing standard operating procedures for pigeon training protocols.
- Responsible for making daily accurate and detailed entries and updates of all work unit laboratory books.
- Responsible for compiling information for and conducting weekly meetings with /between work unit P. I.'s and laboratory technicians.

*Briggs*

- The primary objectives of this period were to continue to improve the efficiency and effectiveness of the toxicity research and to improve the quality of the data collected and presented. This included assisting with project close out reports and new study initiation activities.
- Another key objective was to prepare and review project proposals and pre-proposals to help ensure the NMRI/TD mission is supported and projects are supported by essential research needs.
- Resource allocation and planning efforts were conducted to assess future needs and opportunities. This included dialogue with ONR staff and the Navy veterinarian and veterinary services staff from Brooks AFB and WPAFB.
- The Quality Management Program was launched and the preliminary phase of Standard Operating Procedures and data notebook entries was initiated.



- The Operating Notification 5450 was reviewed, and was issued with clarifications of NMRI/TD staff responsibilities and accountabilities.

*Carraci*

- Submit the Cardiac Sensitization animal use protocol to the Animal Care and Use Committee (ACUC) for approval.
- Coordinate scientific resources for Cardiac Sensitization project.
- Support efforts to continue inhalation experiments of Nonane for the TPH project.

*Connolly*

- Catalog materials as received
- Catalog materials not yet cataloged
- Provide library service to the toxicology community at WPAFB
- Continue working on a manual card catalog

*Garrett*

- Support Dermal Penetration Project
- Conduct *in vivo* dermal exposures of chloropentafluorobenzene (CPFB) in Hairless guinea pigs, Hartley guinea pigs and F344 rats.
- Develop research methods and analytical procedures to determine partition coefficients of perfluorohexyl iodide(PFHI), CPFB, and 1,2-dichlorobenzene (DCB) in skin of three test species.
- Coordinate jugular implantation surgery and dermal exposure experiments
- Set up Gas-Uptake chamber system for determination of the metabolic kinetics of two test chemicals (PFHI and CPFB) in guinea pigs
- Conduct *in vivo* experiments with the Gas-Uptake chamber using PFHI and CPFB.
- Perform duties as Room Monitor for assigned room
- Write and submit abstract for Annual Society of Toxicology meeting before the Oct. 1 deadline.



*Geiss*

- Identify needs in molecular biology research support and design a technical approach to fulfill those needs.
- Develop protocols and research methods for the evaluation of biological effects of Air Force-related materials.
- Cooperate in current research relating to the toxicological effects of trichloroethylene (TCE), its metabolites and other compounds.
- Develop molecular methods for the Predictive Toxicology project.
- Train other scientists in molecular biology research methods.
- Participate in project planning for projects that include molecular biology methods.
- Perform hybridization analysis of RNA from tissues from hyperbaric oxygen experiments.

*Grabau*

Support Species Differences in Skin Penetration:

- Draft journal article for peer-reviewed publication.
- Quantitate epidermal and dermal endpoints following in-vivo exposures.
- Quantitate epidermal and dermal endpoints from human specimens.

Support Combustion Toxicology of Advanced Composite Materials (ACM)

Project:

- Completed and submit jointly authored abstract for 1996 SOT meeting.
- Develop methods to correlate image analysis results from prior burns and newly developed apparatus.

Support Biologically-Based Dose-Response Modeling of Retinoic Acid:

- Completed and submit jointly authored abstract for 1996 SOT meeting.
- Continued support of current research efforts.

Support to Assessment of In-Vivo Biological Effects:

- Develop three-dimensional methods to quantify apoptosis from confocal microscope acquired images.

Support Program Development (Marketing):

- Continued support of the Program Development Strategic Plan.





*Horton, Rix*

- Installation of MS Exchange Server (Enterprise)
- Development of realistic and systematic mechanism for data archiving and preservation
- Continue developing ADP SOP manual
- Implement new Navy RIMS software

*Jung, Narayanan, T.K.*

Trimethylolpropane (TMPP) Evaluation:

- Measurement of the neurotransmitter concentrations in brain homogenate samples
- Continue culturing the neuroblastoma cells for future use in experiments with TMPP and to place a stock of these cells in cryostorage

*Kimmel, Reboulet, Whitehead*

- Given the necessity to relocate laboratory facilities, no technical (research) objectives were to be satisfied this period

*McDougal*

- Continue pharmacokinetic studies in hairless guinea pigs for chloropentafluorobenzene and 1,2-dichlorobenzene.
- Emphasize *in vitro* diffusion cells in the development of the mathematical models for the whole animal experiments.
- Complete organ weight data on Hartley guinea pigs and Fischer 344 rats for physiologically based model.
- Complete development of analytical methods for MACs *in vitro* exposures.

*Narayanan*

- Evaluate neurotoxicity of simulated Persian Gulf War(PGW) environment toxicants in Sprague-Dawley rats
- Detect and quantitate catecholamines and indoleamines in simulated PGW multiple chemicals exposed male rats\* serum
- Measure and quantitate neurotransmitter levels in different regions of brains in control and simulated PGW environment exposed male rats



*Prues, Smith*

SFE Formulations:

- The objective of this research is to evaluate the potential health effects of exposure to the by-products of pyrolyzed SFE. SFE is fire suppressant and a potential replacement for Halon 1301.

Cardiac Sensitization:

- The objective of this research is to develop an *in vitro* test for the determination of cardiac sensitization. These initial studies will set the basic background needed for future studies.

Trimethylpropane phosphate (TMPP):

- The objective of this research is to determine the mechanism of action of TMPP. TMPP is a by-product from the breakdown of synthetic lubricants that produces a neurotoxic response.

Flow injection analysis (FIA):

- The objective of this research is to develop new analytical methods/assays for the detection of toxins.
- Serve as the Contract Representative on the Safety Policy Committee (S. Prues)

*Ritchie*

TMPP MECHANISMS OF ACTION: DEVELOPMENT OF NEUROBEHAVIORAL MOLECULARIZATION TECHNIQUES (WU.1516):

- To complete a major study evaluating the relative capacities of well known human anticonvulsant agents to prevent or counteract neurotoxicity induced by exposure to low or high doses of trimethylolpropane phosphate.
- To develop and test a new system allowing simultaneous recording of electrocorticograms (ECGs) from three free-moving rats.
- To assist in completion of, and present four (5) scientific posters at the 1996 Society for Neuroscience meeting in Washington, DC (Nov 16-21, 1996).
- To prepare and submit five (5) abstracts for the NEHC annual meeting in Virginia Beach, VA in Feb 1997.
- To complete threshold testing of 21 rats implanted with intracranial self-stimulation electrodes (ICSS) for subsequent evaluation following TMPP administration.



Mechanisms Involved with Exposure to Select Neurotoxicants (WU .1712):

- Funding began 01 Oct 1996.
- To begin initial literature search and research planning (in conjunction with Dr. Robert Carpenter).

Development of the Navy Neuro-Molecular Assessment System (the NTAS) [WU .1713]:

- Funding began 01 Oct 1996.
- To work with Dr. Jan Lin in development of methods and techniques for hippocampal tissue slice.
- To work with Dr. T.K. Narayanan for development of methods and techniques for cell culturing of neuroblastoma cells.
- To work with Dr. Eldon Smith for development of quantitative techniques for analysis of neuroprotein markers (i.e., cFOS, GFAP) in response to toxic insult.
- To work with Dr. James Lindsey for development of fast scan cyclic voltammetry techniques.
- To work with Dr. Chip Aucker at NMRI (Bethesda, MD) for literature search and initial planning for programmed research involving histological immunochemistry for neuroprotein analysis following toxic insult.

Neurobehavioral Toxicity Assessment Battery (NTAB): Assessing Animal Responses to Pharmacological Challenge (WU .1605):

- To complete development of auditory startle-pre-pulse inhibition/habituation, photosensitivity, total locomotory behavior, appetitive reinforcer approach conditioning, one-trial passive avoidance, forelimb grip strength and exercise fatigue apparatus and testing methods.
- To work with Dr. Alan Nordholm in development of testing methods for conditioned eyeblink classical conditioning of rats, rabbits and human subjects.
- To procure nine neuroactive chemicals to be used in validation of NTAB tests.
- To complete operant training of 21 pigeons to be used in validation of NTAB tests involving visual discrimination, higher cognitive function and physiologic irritancy.
- To begin planning for development of human tests (i.e., operant conditioning, auditory startle, eyeblink classical conditioning, etc.) to be compared to NTAB tests for predictive validation.



PERSIAN GULF WAR (PGW) SIMULATION USING SPRAGUE-DAWLEY RATS  
(US ARMY AND NMRI/TD):

- To complete the neurobehavioral analysis of (8 tests per rat) of 128 rats exposed to combinations of jet fuel vapor (JP-4), DEET, pyridostigmine bromide and footshock stress.
- To complete a initial study summary for clearance through NMRI, BUMED, the US Army and the Department of Defense, allowing eventual release of research findings.

**SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING  
PERIOD**

*Abbas*

- Submitted PBPK model in mice paper for *Journal of Toxicology and Applied Pharmacology*
- Completed literature review to develop a protocol to investigate the kidney tumor related glutathione pathway of TCE metabolism in rats
- Continued to manage the TCE project, meet the customer needs, and coordinate the TCE research work
- Continued to work on investigation of the role of gut microflora in the TCE metabolism and conversion of TCA to DCA in the gut
- Continued to participate in the TCE inhalation study

*Ademujohn*

- Compiled, organized, and cataloged via computer-aided graphics, the weekly data on Pigeon 'Match' and 'Shapes' protocols.
- Trained and conditioned new and incoming rodent and pigeon groups to protocol adaptation.
- Successfully completed a tutorial seminar for Med-State Notation Computer System Programming.
- Maintenance of all laboratory work unit notebooks
- Implemented several data methods to compile training data and weight maintenance on the pigeon operants.
- Trained summer students in all aspects of laboratory function and procedures



*Briggs*

- Assisted with the preparation and planning of new research pre-proposals for FY 98 funding support from Program 6. Eleven pre-proposals were submitted, ten of which were prepared by GEO-CENTERS, INC., staff members. These submissions were prioritized and two prepared by Dr. Briggs will hopefully receive funding support in FY 97.
- Dr. Briggs supported the O.I.C of NMRI/TD in providing research support for the CENR Endocrine Disruptor Committee activities. CAPT Still is the military representative on this active committee. Dr. Briggs prepared 5 profiles (see Section 5) and reviewed and edited all 26 profiles submitted from the Tri-Service Toxicology Consortium. Dr. Briggs also reviewed the 386 profiles, and was tasked to place the 59 neurotoxicity-related endocrine disruptor profiles into appropriate categories. He also prepared two abstracts relating to endocrine disruptors for the Society of Toxicology and NEHC meeting to be presented next quarter.
- Dr. Briggs reviewed the toxicology characterization reports for HFC 236fa and prepared a summary report of the toxicity studies. This report will be sent to NEHC for establishing exposure standards for this new halon replacement refrigerant.
- Attended 2 MCRA meetings and completed the review of the 20 chemicals of military interest as required for SERDP project support.
- Attended 2 Tri-Service Toxicology Consortium meetings to help with planning and approving the Charter.
- Reviewed the Memorandum of Agreement with the Air force for toxicology and veterinary services support activities. This included a meeting with VS personnel from both the Navy and Air Force. Appropriate comments were submitted to the O.I.C for his report.

*Carraci*

Physiologically Based Pharmacokinetic Modeling of Cardiac Sensitizing Chemicals in Rats:

- Submitted animal protocol to the Armstrong Laboratory Animal Care and Use Committee (ACUC). Protocol was conditionally approved pending the committee's recommendations.
- Animal protocol for this project was modified, and re-submitted to the ACUC.
- Supporting research for project was initiated.



Total Petroleum Hydrocarbon (TPH) Project:

- Gavaged female Fischer-344 rats with Nonane/Soil mixtures. Exhaled air and blood samples were collected for Nonane analysis in order to develop a PBPK model.

*Connolly*

- 42 books cataloged and prepared for circulation
- 146 articles obtained from local libraries
- 5 books borrowed from local libraries for customers here
- 6 interlibrary loans obtained
- 1 interlibrary loan provided to another library
- 7 literature searches conducted using in-house CD-ROM database capabilities
- 5 searches successfully conducted on the internet for customers, including downloading of documents as required
- 12 reference questions answered
- 9 telephone inquiries on journal locations in local area handled successfully
- 33 requests for articles located and filled from in house resources
- 5 articles obtained using the CARL UnCover system via the internet
- 502 card sets prepared for manual card catalog
- 3 orientation trainings and 1 search methods training conducted
- 34 volumes received from bindery and processed in
- 143 journal volumes consulted by customers

*Garrett*

- Worked on skin:air partition coefficient determination of PFHI and CPFB in three test species.
- Began method development for skin:neat chemical partition coefficient determination with PFHI, CPFB, and DCB
- Conducted *in vivo* dermal exposures to CPFB in three test species; Hairless guinea pig, Hartley guinea pig, and F344 rat
- Coordinated jugular implantation surgeries and dermal exposure experiments for test animals.
- Began set-up of new Gas Uptake chamber system.
- Submitted abstract for Annual Society of Toxicology meeting.



*Geiss*

Group Administrator:

- Continued to assist in planning resource allocation, individual training and professional development for the PD group.
- Participated in a team effort to develop an OET Strategic Plan for research in cooperation with the Laboratory management.
- Communicated with other scientists and project leaders to identify their technical needs and to suggest solutions.
- Time was spent on writing/reviewing/editing standard instructions for the lab on the preparation of Standard Operating Procedures (SOP's).
- Participated as a member of the Research Operations Council for OET.
- Initiated a Memorandum of Agreement for potential collaborative research to be performed in conjunction with academic scientists at Miami University (Ohio).

TCE Support:

- Gene probes for target mRNA molecules were prepared and used for further analysis of samples from the TCE study.
- Data obtained from the Northern analysis are being compiled

AFOSR Support:

- Performed literature review for future potential experiments.
- Participated in development of project research plan, including experimental design and study timelines.

Persian Gulf Project:

- Additional samples were obtained and stored for later molecular biology analysis.

Retinoic Acid Project:

- DNA clones for probes to be used in future experiments were prepared, confirmed and stored.

Confocal Microscopy:

- Interacted with project director to establish a research plan.

Laboratory Support:

- Time is being spent on authoring SOP's for molecular biology methods.
- Interact with project directors to facilitate coordination of research resources.



*Grabau*

Support Species Differences in Skin Penetration:

- Journal article for peer-reviewed publication has been drafted and is being reviewed by other authors
- Quantitate epidermal and dermal endpoints following in-vivo exposures were completed.

Support Combustion Toxicology of Advanced Composite Materials (ACM)

Project:

- Completed and submit jointly authored abstract for 1996 SOT meeting.
- Presented methods to correlate image analysis results from prior burns and newly developed apparatus.
- Drafted technical approach for Phase II ACM combustion study.

Support Biologically-Based Dose-Response Modeling of Retinoic Acid:

- Completed and submit jointly authored abstract for 1996 SOT meeting.
- Continue to support of current research efforts.

Support to Assessment of In-Vivo Biological Effects:

- The development of three-dimensional methods to quantify apoptosis from confocal microscope acquired images was initiated by conducting a literature survey. Review of publications is ongoing.

Support Program Development (Marketing):

- Formulation and approval of concepts and design for a Toxicology Division web site resulted in a new web presence. Phased expansion plans have been approved.

*Horton, Rix*

- Successfully completed installation of MS Exchange Server (Enterprise). This state-of-the-art mail system has dramatically improved the E-Mail service to all members of staff as it streamlines local and internet mail services on all workstations.
- Significantly improved Library server performance by bringing 28 bay CD Tower on-line. This system drastically improved seek and retrieval time of scientific journals (MEDLINE and TOXLINE) as well as making other CD-ROMs available to staff, reducing hardware and software costs!





- Avoided significant network shutdown through vigilance and early detection of problem with network memory. An inexpensive solution was achieved through reconfiguring and tripling available RAM on Enterprise Server.
- Initiated three pronged approach of data archiving and preservation through use of on-board tape system and Magneto-Optical system as well as Back-Up Exec software
- Updated Service Packs as needed on Exchange and Network Servers
- Removed outdated MS Mail and Post Office network software
- Increased efficiency of network through removal of two older servers, replacing them with existing hardware enhanced to perform functions of these two older systems
- Installed new server furniture making network administration more efficient
- Completed transfer and setup of 4 personnel to remote locations
- Ordered various software and hardware upgrades
- Continued to reconfigure Windows Browser and WINS for WAN
- Continued conversion of WFW systems to Windows 95
- Continued maintenance of Servers including backing up data files
- Continued support of hardware and software for TOXDET personnel
- Continued to update information Databases
- Continued to update all Internet services (Web, Gopher, Listserv, FTP)
- Continued development of ADP SOP manual - this is an ongoing process that will assist NMRI/TD to meet GALP guidelines
- Identified and corrected problems with animal testing chambers. In addition, developed comprehensive documentation for wiring, functions, and I/O for all laboratory testing chambers. This along with ADP's assistance in the comprehensive reorganization of cabling and set-up of animal chambers, making troubleshooting easier for technicians as well as establishing SOP and trouble log for lab rooms.
- Mr. Horton attended the Microsoft Exchange Server Deployment Conference in Sept 96.
- Mr. Horton attended an in-house workshop on the Med-Associates software and hardware in Nov 96.

*Jung, Narayanan, T.K.*

Trimethylolpropane (TMPP) Evaluation:

- The brains from rats that had been dosed with TMPP (0.5 mg/ kg) or Pentylenetetrazole (60 mg/ kg) and then decapitated immediately after tonic clonic severe grand mal seizures were seen were homogenized after being dissected into cerebellum, brain stem, frontal, middle, and cortical sections. These were frozen in liquid nitrogen and stored at -80 °C. The homogenates were then run under



isocharatic conditions with Citric acid buffer (15 % v/v Methanol) for the detection of the concentration of neurotransmitters. The rat and chicken brains from CDR Rossi were prepared the same way and run under the same conditions.

- The mouse neuroblastoma cell line was removed from cryostorage and put on plates in the incubator with media. The cell line will be cultured and cells periodically stored for future use.

#### Cell model

- This project was put on hold until further funding was received. The liver hepatocyte cells were placed in cryostorage until they will be needed.

#### Publications, Abstracts, etc.

The four pre-proposals written were:

1. Molecular Approaches to Toxicity by Endocrine Disrupters
  2. Peroxisome Proliferation by Endocrine Disrupters
  3. Immunological Alterations by Endocrine Disrupters
  4. Cytochrome P450 Technology in Assessing the Safety and Efficacy of Endocrine Disrupters
  5. Further Development of the NTAS: Cytokines as a biomarker for the processes underlying non-insult performance decrement
  6. Further Development of the NTAS: Steroids and oxidative stress
- The SOT abstract is entitled "Effects of Trimethylolpropane Phosphate on Neurotransmitter Levels in the Rat Brain." A. Jung\*, T. K. Narayanan\*, and J. Rossi III. Naval Medical Research Institute (Toxicology Detachment) \*Geo-Centers, Inc., Wright-Patterson Air Force Base, Oh 45433-7903.
  - The paper entitled "The Effect of a Toxin on Cell Cultures" by D.P. Gaver, R.L. Carpenter, T. K. Narayanan, P.A. Jacobs, and A. Jung was submitted for approval for publication.

#### *Kimmel, Reboulet, Whitehead*

- The work performed for the period is the same as the above description of work.



*McDougal*

Air Force Office of Scientific Research (AFOSR) project (Dermal Penetration):

- Completed pharmacokinetic experiments with chloropentafluorobenzene in Fischer 344 rats and Hairless Guinea pigs.
- Determined skin partition coefficients with tridecafluoroiodohexane and chloropentafluorobenzene.

Modular Artillery Charge System (MACS) project (Dermal Penetration):

- Completed development of analytical methodology for primary components of the propellant (nitroglycerin, diphenylamine, nitroguanidine, 4-aminobiphenyl, dibutylphthalate, nitrocellulose, and dinitrotoluene).
- Completed preliminary *in vitro* studies to measure flux and permeability coefficient of MACS components through "fuzzy" rat skin.

Total Petroleum Hydrocarbon (TPH) project:

- Participated in pilot studies to determine the gastrointestinal absorption of nonane in soil in rats.

Other Scientific and Regulatory interactions:

- Continued to act as scientific mentor for Capt. Wade Weismann with emphasis on laboratory data collection.
- Participated in a working group to recommend methodology for dermal reference concentrations to the US Environmental Protection Agency.

Presentations, Abstracts, etc.

Chapters

McDougal, James N. , Annette L. Bunge, Dermal Uptake (Chapter 6) of ILSI Document on Contaminated Water in Showering and Bathing Scenarios, submitted.

*Narayanan*

Toxicity Evaluation of Simulated Persian Gulf War (PGW) Exposure in Sprague-Dawley Rats, COET324:

- Submitted Standard Operating Procedure (S.O.P) for neurotransmitters\* analysis in different regions of rat\*s brain using HPLC coupled with electrochemical detection
- Control and PGW Chemicals exposed rats\* brains were surgically removed and nine regions of the brains were dissected and stored in freezer for future analysis



- Eluting conditions for neurotransmitters were standardized using new C18 column
- Neurotransmitters' analysis was performed in blood serum and different regions of rat's brain in control and PGW multiple chemicals exposed rats in both 14 days and 60 days exposure studies
- Neurotransmitters and their metabolite levels were quantitated in different regions of rat brain to assess the neurotoxicity of simulated Persian Gulf War(PGW) environment toxicants using HPLC in both 14 days and 60 days exposure studies
- Reprocessed data for all the control and simulated PGW environment toxicants exposed samples
- Statistically significant differences in neurotransmitter levels between control and simulated PGW environment exposure groups were estimated using student's t-test for unpaired data

Other Duties:

- Literature survey was done to support future hormone and combustion studies

Waste Management:

- Attended annual "Resource Conservation Recovery Act (RCRA)" training course

Presentations, Publications, Abstracts, etc.

- K MacMahon, J Rossi, R E Wolfe, J. H. King, L Narayanan, F Witzmann, J Ritchie and A Nordholm. Physiological Parameters Of Sprague-Dawley Rats Exposed To Low Doses Of Pyridostigmine Bromide, Deet, JP-4 Jet Fuel And Stress. (Society Of Toxicology abstract 1996.)

*Prues, Smith*

SFE Project

- Organization of data books for SFE range-finding/multiple-dose, edema and homeostasis studies. Submitted final drafts for technical reports on SFE Formulation A pilot studies: Part I and Part II.
- SFE Project -- Homeostasis
- Editorial support for publications.
- Acquired specialized equipment to perform the necessary surgery for this project.
- Surgical techniques/ hardware worked out for procedure
- Testing of animals begun.



TMPP Project -- Glial Fibrillary Acidic Protein (GFAP)

- Additional acquisition and review begun of GFAP articles for possible Review article.
- Inventoried/ordered supplies necessary for ELISA assay.

FIA/SIA Project-- Cyclic Voltametry

- Read operation manuals on system hardware and software
- Ran various procedures for familiarization and testing of system
- Instituted reconfiguration of system to correct problems
- Currently testing support hardware (florescence detector)

Cardiac Sensitization Project

- Write new cardiac sensitization protocols to evaluate mechanical parameters in swine.
- Several cardiac sensitization studies were initiated to study the mechanical and electrophysiological events leading to ventricular fibrillation.

*Ritchie*

TMPP MECHANISMS OF ACTION: DEVELOPMENT OF NEUROBEHAVIORAL MOLECULARIZATION TECHNIQUES (WU .1516):

- Completed a major study evaluating the relative capacities of well known human anticonvulsant agents (valproic acid, ethosuximide, diazepam and phenobarbital) to prevent or counteract absence-like seizures to generalized motor seizures induced by exposure to low or high doses of trimethylolpropane phosphate.
- Developed a new system allowing simultaneous recording of electrocorticograms (ECGs) from three free-moving rats.
- Presented four (5) scientific posters at the 1996 Society for Neuroscience meeting in Washington, DC (Nov 16-21, 1996).
- Submitted five (5) abstracts for the NEHC annual meeting in Virginia Beach, VA in Feb 1997.
- Completed threshold testing of 21 rats implanted with intracranial self-stimulation electrodes (ICSS) for subsequent evaluation following TMPP administration.

Mechanisms Involved with Exposure to Select Neurotoxicants (WU .1712):

- Completed initial literature survey, emphasizing neurobehavioral consequences of exposure of animals or humans to low levels of jet fuels, gasoline and kerosene.



Development of the Navy Neuro-Molecular Assessment System (the NTAS) [WU .1713]:

- Assisted in completion of hippocampal tissue slice methods.
- Assisted in successful cell culturing of neuroblastoma cells.
- Assisted in ordering of supplies for neuroprotein analysis.
- Worked extensively with NMRI/TD Radiation Safety Officer to procure two radioactive substance licenses required for completion of TMPP ligand binding studies; assisted in analysis of data reflecting regional binding of labeled TMPP at the time of seizure onset.

Neurobehavioral Toxicity Assessment Battery (NTAB): Assessing Animal Responses to Pharmacological Challenge (WU .1605):

- Completed development of auditory startle-pre-pulse inhibition/habituation, photosensitivity, total locomotory behavior, appetitive reinforcer approach conditioning, one-trial passive avoidance, forelimb grip strength tests.
- Developed testing methods for conditioned eyeblink classical conditioning of rats, rabbits and human subjects.
- Procured nine neuroactive chemicals to be used in validation of NTAB tests.
- Completed operant training of 21 pigeons to be used in validation of NTAB tests involving visual discrimination, higher cognitive function and physiologic irritancy.
- Identified testing laboratories (San Diego Naval Medical Hospital, etc.) for conducting human tests to be compared to NTAB tests for predictive validation.

PERSIAN GULF WAR (PGW) SIMULATION USING SPRAGUE-DAWLEY RATS (US ARMY AND NMRI/TD):

- Completed testing (eight neurobehavioral tests) of 128 rats exposed for 14 days to a simulated Persian Gulf war environment.
- Submitted initial research report to DoD.

Presentations, Publications, Abstracts, etc.

- J.W. Lindsey, S.L. Prues, C. Alva, G.D. Ritchie and J. Rossi III. Trimethylolpropane phosphate microperfusion into the nucleus accumbens of rat: electroencephalic, behavioral and neurochemical correlates. Submitted for publication, NeuroToxicology, Oct 1996.
- G.D. Ritchie, J. Rossi III, A. Nordholm, C.Y. Ademujohn, C. Onyika, J. Smith and A. Walsh. Protection against absence-like and generalized seizures induced by the organophosphate trimethylolpropane phosphate. Society for Neuroscience Abstracts, Vol 22 (3), 820.4.



- J. Rossi III, M.Y. Bekkedal, B. Knutson, G.D. Ritchie and J. Panksepp. Long-term behavioral sensitization induced by a bridged organophosphate. Society for Neuroscience Abstracts, Vol 22 (3), 820.5.
- J.W. Lindsey, S.L. Prues, C. Alva, G.D. Ritchie and J. Rossi III. TMPP microperfusion into the nucleus accumbens of the rat: neurobehavioral and neurochemical effects. Society for Neuroscience Abstracts, Vol 22 (3), 820.6.
- J. Lin. J. Cassell, G.D. Ritchie and J. Rossi III. Repeated exposure to trimethylolpropane phosphate induces mesolimbic dopamine system sensitization in male rats. Society for Neuroscience Abstracts, Vol 22 (3), 820.7.
- G.D. Ritchie, J. Rossi III, A. Nordholm, C.Y. Ademujohn, C. Onyika, J. Smith and A. Walsh. Protection against absence-like and generalized seizures induced by the organophosphate trimethylolpropane phosphate. Society for Neuroscience Poster Presentation, 21 November 1996, Washington, DC.
- J. Rossi III, M.Y. Bekkedal, B. Knutson, G.D. Ritchie and J. Panksepp. Long-term behavioral sensitization induced by a bridged organophosphate. Society for Neuroscience Poster Presentation, 21 November 1996, Washington, DC.
- J.W. Lindsey, S.L. Prues, C. Alva, G.D. Ritchie and J. Rossi III. TMPP microperfusion into the nucleus accumbens of the rat: neurobehavioral and neurochemical effects. Society for Neuroscience Poster Presentation, 21 November 1996, Washington, DC.
- J. Lin. J. Cassell, G.D. Ritchie and J. Rossi III. Repeated exposure to trimethylolpropane phosphate induces mesolimbic dopamine system sensitization in male rats. Society for Neuroscience Poster Presentation, 21 November 1996, Washington, DC.
- Jan Lin, Jeffrey Cassell, Glenn Ritchie and John Rossi III. REPEATED EXPOSURE TO TRIMETHYLOLPROPANE PHOSPHATE (TMPP) INDUCES MESOLIMBIC DOPAMINE SYSTEM SENSITIZATION IN MALE RATS. Abstract, The 38th Navy Occupational Health and Preventive Medicine Workshop, Feb 1997, Virginia Beach, VA.
- JOHN Rossi III, MARNI .Y. Bekkedal, BRIAN Knutson, GLENN D. Ritchie and JAAK Panksepp. LONG-TERM BEHAVIORAL SENSITIZATION INDUCED BY A BRIDGED ORGANOPHOSPHATE. Abstract, The 38th Navy Occupational Health and Preventive Medicine Workshop, Feb 1997, Virginia Beach, VA.
- JEFFREY CASSELL, MIKE HOLMES, Glenn Ritchie and CYNTHIA ONYIKA. THE ROLE OF THE NAVY HOSPITAL CORPSMAN AT THE TRI-SERVICE TOXICOLOGY CONSORTIUM AT WRIGHT-PATTERSON AFB. Abstract, The 38th Navy Occupational Health and Preventive Medicine Workshop, Feb 1997, Virginia Beach, VA.
- GLENN D. Ritchie, JOHN Rossi III, ALAN Nordholm, JEFFREY Cassell and JAY Smith. Protection against absence-like seizures induced by the organophosphate



trimethylolpropane phosphate. Abstract, The 38th Navy Occupational Health and Preventive Medicine Workshop, Feb 1997, Virginia Beach, VA.

- JAN Lin, JEFFREY Cassell, GLENN RITCHIE AND JOHN Rossi III. REPEATED EXPOSURE TO TRIMETHYLOLPROPANE PHOSPHATE (TMPP) INDUCES MESOLIMBIC DOPAMINE SYSTEM SENSITIZATION IN MALE RATS. Abstract, The 38th Navy Occupational Health and Preventive Medicine Workshop, Feb 1997, Virginia Beach, VA.

### **GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD**

#### *Abbas*

- Finish remaining duties and provide customer with all scientifically relevant material and information. Last day of employment with GEO-CENTERS, INC.. will be October 18, 1996

#### *Ademujohn*

- To accurately and efficiently compile, log organize and analyze all incoming data from inhalation studies.
- To accurately train rodents for various testing protocols, such as EEG, swim test.
- To accurately train pigeons for upcoming testing protocols for upcoming drug studies.
- To successfully work with rabbits for upcoming neurobehavioral studies.
- To maintain a clean and orderly laboratory environment.
- To provide technical support in testing relative toxicity of various pharmaceuticals in pigeons and rats and rabbits.
- To procure and document pigeon maintenance pertaining to preparatory requirements for 'shaping' activities , pre-testing and testing protocols.

#### *Briggs*

- Continue to support the current research projects and assure that contractor resources are available.
- Continue to perform reviews of Standard Operating Procedures and critical phases of current research projects
- Present endocrine disruptor papers and/or posters at the Society of Toxicology (SOT) and NEHC meetings. Attend the Society of Risk Analysis Meeting including the Risk





Communication Workshop. Also attend the SOT and NEHC meetings and an Endocrine Disruptor meeting.

- Meet with the HFC 236fa committee to finalize the toxicology characterization study data so this new refrigerant will be used by the Navy aboard ships.
- Continue to work on the MCRA committee to review toxic chemicals of interest to the military.
- Participate in the Spring Conference as tasked.

*Carraci*

- Receive final approval of Cardiac Sensitization animal use protocol from the ACUC and the Surgeon General's Research Oversight Committee (SGROC).
- Begin non-animal laboratory experiments for Cardiac Sensitization project.
- Continue to support laboratory experiments for TPH project.

*Connolly*

- Continue cataloging
- Continue preparing cards for the manual card catalog
- Continue training program

*Garrett*

- Continue to provide support to Pharmacokinetics Group and Dermal Penetration Project.
- Complete dermal exposures of CPF in all three test species.
- Continue to coordinate and perform rodent surgeries for Dermal Penetration Project and other Pharmacokinetics Group projects as necessary.
- Begin dermal exposures of DCB in F344 rats, Hartley guinea pigs, and Hairless guinea pigs.
- Conduct Gas Uptake experiments
- Complete partition coefficient experiments
- Work on poster presentation for Society of Toxicology Meeting.

*Geiss*

- Continue to develop molecular methods for use in our lab.
- Integrate new technologies into the battery of methods used to support Toxicology Division projects.



- Continue to perform assigned Group Administrator tasks.
- Continue to assist in training scientists in molecular methods.
- Continue to develop probes for use in hybridization experiments.
- Prepare a technical report for the TCE project.

*Grabau*

Support Species Differences in Skin Penetration:

- Complete journal article for peer-reviewed publication.
- Quantitate epidermal and dermal endpoints following in-vivo exposures.
- Quantitate epidermal and dermal endpoints from human specimens.

Support Combustion Toxicology of Advanced Composite Materials (ACM) Project:

- Co-present abstract at 1996 SOT meeting.
- Utilize recently developed methods to correlate image analysis results from prior burns and newly developed apparatus.

Support Biologically-Based Dose-Response Modeling of Retinoic Acid:

- Present abstract at 1996 SOT meeting.
- Continued support of current research efforts.

Support to Assessment of In-Vivo Biological Effects:

- Develop three-dimensional methods to quantify apoptosis from confocal microscope acquired images.

Support Program Development (Marketing):

- Develop new presentation materials for conferences, laboratory visits and web site.

*Horton, Rix*

- Develop new Supply Database using existing software (Microsoft Access)
- Provide guidance and training in Corel Draw and Power Point to Admin Assistant Office, thereby enabling that office to better support posters and presentations created by scientific staff
- Continue developing ADP SOP manual
- Update all internet services, to include new information for NMRI TOXDET Web Page, as well as further optimizing Gopher, Listserv and FTP



- Identify and replace systems that are not compatible with Office 95 in order to fully implement Microsoft Office Professional
- Continue migration to new office suite software - Microsoft Office Professional
- Mr. Rix should attend a Microsoft Exchange or SMS/SQL course
- Mr. Horton should attend a Network Security course

*Jung, Narayanan, T.K.*

- To increase the productivity in the lab
- To continue the TMPP binding studies
- To begin human cortical neurons for experiments with TMPP
- To continue the work with the rat liver cells of the cell model project
- To finish HPLC analysis of the neurotransmitter samples
- To culture and store neuroblastoma cells for experiments with TMPP

*Kimmel, Reboulet, Whitehead*

- Complete installation of inhalation exposure systems (two whole-body and one nose-only inhalation exposure chamber) to include all control, generation, and atmosphere analysis subsystems. Installation will include all appropriate calibrations. Complete installation of first stage (fundamental measurements ventilation, respiratory mechanics, and gas exchange - approximately 25 % of parameter determination) of small animal pulmonary function testing laboratory.
- Complete protocol development for research program to investigate smoke inhalation induced Acute Lung Injury (ALI) and ARDS.
- Initiate data reduction and analysis for a reactivated inhalation toxicity investigation conducted under the auspices of another contractor. (Acrolein - aerosol particle interaction and effects on pulmonary toxicity). Research germane to development of an animal model of ALI and to be presented at this years annual Society of Toxicology Meeting.
- Prepare a fundamentals of ARDS presentation to be given by NMRI/TD commander.
- Design and build non-rebreathing inhalation exposure system for large animals (dogs and pigs) in support of Cardiotoxicity research program.

*McDougal*

- none - final report for this contract



*Narayanan*

- Measure and quantitate neurotransmitters and their major metabolite levels in control and Triaryl Phosphate exposed rats using HPLC coupled with electrochemical detection
- Measure and quantitate neurotoxic esterase and acetylcholine esterase enzyme levels in different regions of the rat brain in control and Triaryl Phosphate exposed rats
- Support the Toxicity Evaluation Simulated Persian Gulf War (PGW) Exposure in Sprague-Dawley Rats project
- Support the Toxicity Evaluation Of Combustion atmospheres generated from Advanced Composite Materials (ACM) in Sprague-Dawley Rats project
- Identify major toxicants and make multiple assessment of physiological parameters such as blood gases, methemoglobin and carboxyhemoglobin in blood samples drawn from control and combustion atmosphere exposed animals
- Measure and Evaluate toxicity of hydrogen cyanide in blood samples drawn from control and combustion atmosphere exposed animals
- Measure and quantitate neurotransmitters and their major metabolite levels in control and PGW multiple chemicals exposed rats using HPLC coupled with electrochemical detection.
- Detect and quantitate Nonane in rat tissues exposed to different concentrations of Nonane by inhalation, using headspace GC, with on line Flame Ionization Detector (FID)
- Continue ongoing research projects by repeating some of the experiments and being involved in research projects that are of interest to Tri-Service

*Prues, Smith*

- Initiate pulmonary physiology studies on aerosol effects on the lung.
- Begin writing new protocols in the area of pulmonary toxicology.
- Begin developing GFAP assay for the neurobehavioral group to investigate subtle changes in the central nervous system.
- Close data books for SFE range-finding/multiple-dose, edema and blood gas studies.
- Surgical and technical support for the Homeostasis portion of the SFE project.
- Technical support for the GFAP immunoassay portion of the TMPP project
- Provide continued technical support for the characterization of the SIA system used on the FIA project



*Ritchie*

TMPP MECHANISMS OF ACTION: DEVELOPMENT OF NEUROBEHAVIORAL MOLECULARIZATION TECHNIQUES (WU .1516):

- Evaluate the relative capacities of well known human anticonvulsant agents (valproic acid, ethosuximide, diazepam and phenobarbital), combined with a GABA<sub>B</sub> antagonist to prevent or counteract absence-like seizures induced by exposure to doses of trimethylolpropane phosphate.
- Present five (5) abstracts for the NEHC annual meeting in Virginia Beach, VA in Feb 1997.
- Complete testing of 21 rats implanted with intracranial self-stimulation electrodes (ICSS) following TMPP administration.

Mechanisms Involved with Exposure to Select Neurotoxicants (WU .1712):

- Begin initial research planning for exposure of rats to jet fuels.

Development of the Navy Neuro-Molecular Assessment System (the NTAS) [WU .1713]:

- Use of tissue slice methods to evaluate TMPP effects on single unit response in the rat hippocampus.
- Use of neuroblastoma cell cultures to evaluate effects of TMPP on cell development and differentiation, membrane integrity and apoptosis.
- Assistance in initiation of neuroprotein assay techniques.
- Completion of TMPP ligand binding studies.

Neurobehavioral Toxicity Assessment Battery (NTAB): Assessing Animal Responses to Pharmacological Challenge (WU .1605):

- Development of juvenile play behavior, Morris Water Maze and Porsolt Forced Swim tests.
- Initial conditioned eyeblink classical conditioning of rabbits and human subjects.
- Use of nine neuroactive chemicals for validation of five NTAB tests using rats.
- Completion of operant training of 21 pigeons to be used in validation of NTAB tests involving visual discrimination, higher cognitive function and physiologic irritancy.



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January 31, 1997

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PERSIAN GULF WAR (PGW) SIMULATION USING SPRAGUE-DAWLEY RATS  
(US ARMY AND NMRI/TD):

- Publication of the results of the PGW simulation study.
- Submission of a \$2 MM proposal to the DoD to continue the PGW simulation study.



GEO-CENTERS, INC.

V. NMRI, Natick, MA

A. HUMAN PERFORMANCE AND U.S. NAVY CLOTHING DEVELOPMENT

DESCRIPTION OF WORK TO BE PERFORMED

*Lacerte, Macek, Pawar, Schneider, Buller, Burke,  
Meyers, Monarrez, Donaldson, Collins, Madden,  
Grafton, Reynolds, Smith S., Smith/PM, Kubler*

Program I: Flame Protective Clothing Research

- The math modeling project involved work at the University of Texas on mathematical simulation of human thermal behavior using whole body model. The detail discussion of this approach is found in the 'Heat transfer in medicine and biology: analysis and applications.' The main tasks in this project were to understand the working of the FORTRAN program software, its input parameters, learn the method of creating input file, and execute the simulation runs to get meaningful results.
- Evaluation of response of copper calorimeter at various heat fluxes on the automated TPP Test equipment was another research objective. This task is a continuation of the Thermal Protection Analysis System previously developed. The TPA system can be used as a convenient method for calibrating copper calorimeters to satisfy second-degree burn criterion developed by Stoll et al. It was required to develop an extension for the heat gun to keep it at a fixed distance from the surface under heating.
- Finite vapor pressure and latent heat of vaporization have been used in microclimate cooling. The cooling of garments on an equal basis i.e., independent of microclimate cooling systems is being investigated. Performance equations for such garments are also being developed. The research interest created a need for development of data acquisition system to measure temperatures, pressures and flow rates in experiments.

Program II: U.S. Navy Certification Program for Commercial  
Environmental/Occupational (CEO) Protective Clothing/Equipment

- GEO-CENTERS, INC.. will establish a program to be used by NCTRF to certify commercial off-the-shelf protective clothing/equipment as meeting or exceeding Navy functional performance requirements. This program will make possible the direct purchase of certified commercial protective clothing/equipment for shipboard use by Navy personnel.



GEO-CENTERS, INC.

Program III: Database Search

- Conduct an extensive search of databases to determine commercial, DoD and non-DoD government organizations with which the U.S. Navy Clothing & Textile Facility (NCTRF) may enter into cooperative R&D agreements for the research, development, and testing of dress and protective clothing systems.
- Determine cooperative opportunities for dual-use technology, technology transition, and technology exploitation.
- Prepare a technical briefing to highlight the technical expertise and unique facilities and equipment available at NCTRF. This briefing could be exploited by agencies seeking cooperative research, development, and acquisition agreements.
- Prepare documentation to convey the technical expertise and unique facilities and equipment available at NCTRF. This documentation could be exploited by agencies seeking cooperative research, development, and acquisition agreements.

Program IV: Great Lakes Prototype Footwear Test

- Provide technical support in the development of the Enhanced Chukka Shoe surveys for recruits, leaders, shipboard personnel, and Naval Academy personnel.
- Provide technical support for experimental design of study.
- Provide software support in the production of an on-line data entry program and database management.
- Provide data collection support at the Recruit Training Center (RTC).
- Analyze data by test group and write final report of findings of the study.

Program V: Technical Reports

- Analyze and organize information provided on projects conducted in the Navy Clothing and Textile Research Facility (NCTRF).
- Develop technical reports and articles for publication in peer-reviewed journals.

Program VI: Commercial Off-the-Shelf Utility Uniform Study (COTS) and The Utility Uniform Study

Subtask 1: Commercial Off-the-Shelf Utility Uniform Study (COTS)

- Design questionnaire to assess fit, performance, durability and preference for two commercial off-the-shelf utility uniforms. The two styles are: 1) Redcap, and 2) Levi 505.
- Produce issue data sheets and explanatory package for subjects
- Reproduce questionnaires and issue packages.





Subtask 2: Utility Uniform Study

- Adapt questionnaire, data sheets, and explanatory package from subtask 1 for three uniform configurations: 1) 14 oz. Denim with 4 oz. Chambray Shirt, 2) 11 oz. Denim with 4 oz. Chambray Shirt, and 3) "Dickie" Style.
- Reproduce questionnaires and issue packages for all test participants.
- Provide support of 2 issuers to 16 test sites on the East and West Coasts, with approximately 75 test participants at each site.
- Provide support of two Human Factors Engineers to visit each test site twice during the duration of the study to issue and collect surveys and to collect subjects' comments. Visits will occur three and six months after issue of utility uniforms.
- Enter, clean, verify, and tabulate collected data.
- Analyze data, based upon experimental design and study hypothesis, using standard univariate and multivariate statistical techniques.
- Prepare a report detailing the whole study providing a clear explanation of the analytical techniques adopted and the conclusions reached from analysis of the data.

**TECHNICAL OBJECTIVES FOR THE REPORTING PERIOD**

*Lacerte, Macek, Pawar, Schneider, Buller, Burke,  
Meyers, Monarrez, Donaldson, Collins, Madden,  
Grafton, Reynolds, Smith S., Smith/PM, Kubler*

Program I: Flame Protective Clothing Research

The major research goals for the current reporting period were to:

- Study the predictive capability of the Texas Human Thermal Model (also known as the Wissler Model) during cold water immersion for given garments.
- Design a method and equipment for calibration of copper calorimeter under various heat exposure levels.
- Search for appropriate manikin diagram and software tools to control the properties of sensor areas in the diagram.
- Design graphic user-interface for the microclimate cooling system.

Program II: U.S. Navy Certification Program for Commercial  
Environmental/Occupational (CEO) Protective Clothing/Equipment

- Upon receiving comments from NCTRF on the certification program report, GEO-CENTERS, INC.. will incorporate the changes into the report.



Program III: Database Search

- The modular briefing will be completed.
- A high quality color brochure, which outlines the NCTRF capabilities, product developments, and specialized laboratories and equipment will be completed.

Program IV: Great Lakes Prototype Footwear Test

- None. The final technical report was completed and has been submitted to NCTRF.

Program V: Technical Reports

- Finalize and submit the following two reports to NCTRF:
  - "Development of a Rough Sea Simulation Method for Testing Protective Clothing"
  - "Validation of Rough Sea Simulation Methods for Testing Protective Clothing"
- Continue to make editorial changes and incorporate comments from NCTRF into the following report:
  - "Correlation of Thermal and Evaporative Resistances of Military Clothing Items, Measured on a Guarded Hot Plate and Thermal Manikin"

Program VI: Commercial Off-the-Shelf Utility Uniform Study (COTS) and The Utility Uniform Study

- Produce questionnaire for COTS study.
- Produce issue data sheets, and package explaining study.
- Reproduce questionnaire, data sheets and subject package for the issue phase of the COTS study.
- Adapt questionnaire, data sheets, and subject package for use with the utility uniform study.

**SUMMARY OF WORK PERFORMED DURING CURRENT REPORTING PERIOD**

*Lacerte, Macek, Pawar, Schneider, Buller, Burke,  
Meyers, Monarrez, Donaldson, Collins, Madden,  
Grafton, Reynolds, Smith S., Smith/PM, Kubler*

Program I: Flame Protective Clothing Research

- The data on human subjects wearing the anti-exposure suit and float coat under cold water immersion were obtained from technical reports. Since the data was in graphical form, the numerical data had to be generated from its graphical representation in these



reports. This data was written to an acceptable file format for the program and the model was run for given parameters related to the human subject and his environment. The model predictions were analyzed and summarized.

- The thermocouple connectors and other hardware were purchased for evaluation of response of a copper calorimeter sensor. The design of an extension for the heat gun for the sensor evaluation purpose was discussed. The schematic of the extension part was made and submitted for fabrication.
- All in-house mountings for exposure of skin simulant sensor were studied. These mountings can be used for calibration of sensor and or heat source without having to modify them.
- Research was performed on the relationship between the degree of burn injury and the probability of survival of a victim. Research was also performed on manikin diagram and an appropriate file format for such diagram. National Instruments suggested to use "Canvas Control" from their software development package for creation of controls for manikin diagram.
- A prototype of graphic user interface (GUI) for evaluation of microclimate cooling systems used in manikin tests was created. The in-house pressure transducers, flow rate transducers and circulating chiller for interfacing with computer was studied. The work on development of hardware interface for this system is in progress.

Program II: U.S. Navy Certification Program for Commercial  
Environmental/Occupational (CEO) Protective Clothing/Equipment

- Awaiting comments from NCTRF on the draft report.

Program III: Database Search

- Completed scanning 174 photos and delivered disks containing scanned material.
- Delivered a complete set of colored briefing vu-graphs (94 vu-graphs).
- Delivered a complete set of colored hard copies of the briefing (94 vu-graphs).
- Delivered disks which contained the briefing (94 vu-graphs). These disks will be used with a CPU to project the briefing through a video projector.
- Developed colored artist drawings of the Traversing Instrumented Manikin and the Hydro-Environmental Simulator.
- Initiated preparation of a high quality brochure.



Program IV: Great Lakes Protective Footwear Test

- None. The final technical report was completed and has been submitted to NCTRF.

Program V: Technical Reports

- Work was completed on two technical reports for the Navy Clothing and Textile Research Facility (NCTRF) and final bound copies were submitted to the project officer. The reports deal with the development of rough sea simulation conditions in the NCTRF hydro-environmental tank. The first of the two reports, *Development of a Rough Sea Simulation Method for Testing Protective Clothing* was concerned primarily with the comparative effectiveness of eight different methods of water agitation in accelerating the heat loss from a thermal manikin positioned in the NCTRF hydro-environmental tank. The goal was to develop a method suitable for testing with human subjects which could replace the more expensive and hazardous ocean testing of immersion protection clothing systems. The second technical report, *Validation of Rough Sea Simulation Methods for Testing Protective Clothing: Comparison of Field and Laboratory Test of Body Cooling Rates*, is concerned with the results of tests carried out with volunteers under ocean and laboratory conditions. The laboratory conditions were based on variations of a diffused compressed air method of water agitation in the NCTRF hydro-environmental tank. As part of the study a comparison was also made of the immersion protection performance of three clothing systems.
- The review of the draft copies of a third technical report, *Correlation of Thermal and Evaporative Resistances of Military Clothing Items, Measured on a Guarded Hot Plate and Thermal Manikin*, was completed by NCTRF and the changes and corrections requested by the project officer were incorporated in the document. In addition, changes were made in order to arrive at a more consistent designation of the various quantities used to describe thermal and evaporative heat transfer. The manuscript is complete, but consideration is being given to the possibility of adding figures illustrating the six clothing systems and the thermal manikin and guarded hot plate instrumentation.

Program VI: Commercial Off-the-Shelf Utility Uniform Study (COTS) and The Utility Uniform Study

- Questionnaire for COTS study was designed and reproduced.
- Data sheets for COTS study were produced along with the subject package.



- Questionnaires, data sheets, and subject package were reproduced for issue in COTS study.
- Questionnaire, data sheets, and subject package were adapted and redesigned for use in the utility uniform study.

### **GOALS/OBJECTIVES FOR NEXT REPORTING PERIOD**

*Lacerte, Macek, Pawar, Schneider, Buller, Burke,  
Meyers, Monarrez, Donaldson, Collins, Madden,  
Grafton, Reynolds, Smith S., Smith/PM, Kubler*

#### Program I: Flame Protective Clothing Research

- In view of the "sweating thermal foot project" kick off meeting on Dec. 20, 1996, acquisition of TPP hardware and the ongoing math modeling project following research goals are scheduled for the first quarter of 1997.
1. Develop a method to characterize thermal response of a copper calorimeter and that of a sensor with embedded thermocouple.
  2. Develop a prototype data acquisition system for above method.
  3. Learn operation of thermal foot and provide programming guidance to the project team.
  4. Validate Wissler math model on basis of data from other garment types.

#### Program II: U.S. Navy Certification Program for Commercial Environmental/Occupational (CEO) Protective Clothing/Equipment

- Upon receiving comments from NCTRF on the certification program report, GEO-CENTERS, INC. will incorporate the changes into the report.

#### Program III: Database Search

- Deliver a complete set of 35mm slides of the briefing (94 vu-graphs)
- Develop and deliver a high quality reproducible color brochure which outlines the NCTRF capabilities, product developments, and specialized laboratories and equipment.



Program IV: Great Lakes Protective Footwear Test

- None

Program V: Technical Reports

- Complete the final editing and publication of the one pending technical report.

Program VI: Commercial Off-the-Shelf Utility Uniform Study (COTS) and The Utility Uniform Study

- Reproduce questionnaires, data sheets and subject issue package for the utility uniform study.
- Visit test sites on the West and East coast and aid in the issue of the uniforms.
- Enter issue, clean, and validate issue data.



# APPENDIX



*GEO-CENTERS, INC.*





## CELL BIOLOGY OF HYPOXIA - 1996

### Editors:

T. B. Nielsen

J. L. Kidwell

Naval Medical Research and  
Development Command  
Bethesda, Maryland 20889-5606

Bureau of Medicine and Surgery  
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# **CELL BIOLOGY OF HYPOXIA, 1996**

September 9 and 10, 1996

Gaithersburg, Maryland

Organized By:

T.B. Nielsen and A. Murphy

Edited By:

T.B. Nielsen and J.L. Kidwell

Sponsored By:

Office of Naval Research

Hosted By:

Naval Medical Research Institute

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The opinions and assertions contained herein are the private ones of the speakers and editors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals." Institute of Laboratory Animals Resources, National Research Council, DHHS, Publication No. (NIH) 86-23 (1985).

The work was supported by NMRDC Work Unit No. 63706N M095.001-1532.

## Schedule

September 9, 1996

8:00-8:05    **Introduction:**    Dr. Anne Murphy, Department of Biochemistry & Molecular Biology, George Washington University Medical Center

8:05-8:10    **Welcome:**    Thomas Contreras, CAPT, MSC USN, Commanding Officer, Naval Medical Research Institute

8:10-8:15    **Statement of Navy Goals:**    Dr. Constance Oliver, Biomedical Science and Technology, Office of Naval Research

### **Session I: Biological Sensing of Oxygen in Hypoxia**

Chairperson: Dr. Dean Jones, Department of Biochemistry, Emory University

8:15-8:25    **Short background**  
Dr. Dean Jones, Department of Biochemistry, Emory University

8:25-9:05    **Characterization of the master regulator of oxygen sensing and utilization - the heme activating protein 1**  
Dr. Li Zhang, Department of Biochemistry, New York University Medical Center

9:10-9:50    **Integration of sensing mechanisms for rapid and chronic hypoxia**  
Dr. Dean Jones, Department of Biochemistry, Emory University

9:55-10:10    **BREAK**

### **Session II: Cellular Responses to Hypoxia**

Chairperson: Dr. John Lemasters, Department of Cell Biology and Anatomy, University of North Carolina School of Medicine

10:10-10:20    **Short background**  
Dr. John Lemasters, Department of Cell Biology and Anatomy, University of North Carolina School of Medicine

### **Subsession on Metabolic Homeostasis**

10:20-11:00    **Insights provided by glycine cytoprotection into cellular mechanisms of hypoxic injury**  
Dr. Joel Weinberg, Nephrology Research, University of Michigan Medical Center

11:05-11:45    **Bcl-2 potentiation of mitochondrial Ca<sup>2+</sup> uptake capacity**  
Dr. Anne Murphy, Department of Biochemistry & Molecular Biology, George Washington University Medical Center

- 11:50-12:30**   **Prevention of pH-dependent reperfusion injury**  
Dr. John Lemasters, Department of Cell Biology and Anatomy, University of North Carolina School of Medicine
- 12:35-1:40**   **LUNCH**
- 1:40-2:20**   **Subsession on Transcription**  
**Expression of heat shock proteins during hypoxia and exogenous stress**  
Dr. Alexander Murashov, Health Sciences, Columbia University
- 2:25-3:05**   **Expression of stress glycoproteins and heat shock proteins in renal proximal tubule cells after transient hypoxia**  
Dr. Kurt Henle, Medical Research, Veterans Administration Medical Center
- 3:10-3:25**   **BREAK**
- 3:25-4:05**   **Subsession on Effector Systems**  
**Role of endonuclease in hypoxic injury**  
Dr. Sudhir Shah, Division of Nephrology, University of Arkansas for Medical Sciences
- 4:10-4:50**   **Calpain proteases as an effector mechanism in hepatocyte necrosis during anoxia**  
Dr. Gregory Gores, Center for Basic Research in Digestive Diseases, Mayo Clinic

# Cell Biology of Hypoxia, 1996

September 10, 1996

8:00-8:05 Greeting

## Session II (continued)

### Subsession on Responses of Endothelial Cells

8:05-8:45 Human umbilical vein endothelial cells (HUVEC) are resistant to hypoxia-induced apoptosis and have increased expression of mRNA for cytoprotective molecule A1

Dr. Robert Winn, Department of Surgery, University of Washington

8:50-9:30 Differential mechanisms of death in cerebral endothelial cells: hypoxia, hyperoxia, and inflammatory signals

Dr. Chung Hsu, Cerebrovascular Disease Section, Washington University School of Medicine

9:35-9:50 BREAK

## Session III: Animal Models and Treatments

Chairperson: Dr. Ken Proctor, Department of Physiology and Biophysics, University of Tennessee

9:50-10:00 Short background

Dr. Ken Proctor, Department of Physiology and Biophysics, University of Tennessee

10:00-10:40 The role of IL-6 in gut reperfusion following hemorrhage

Dr. Florence Rollwagen, Resuscitative Medicine Program, Naval Medical Research Institute

10:45-11:25 Trauma and hemorrhage alter *in vivo* cell proliferation, gene expression, and apoptosis

Dr. Thor Nielsen, Resuscitative Medicine Program, Naval Medical Research Institute

11:30-12:10 Development of clinically-relevant models of traumatic shock

Dr. Ken Proctor, Department of Physiology and Biophysics, University of Tennessee

12:10-1:20 LUNCH

## Session IV: Assessment

1:20-1:25 Introduction of the Panel

Dr. Thor Nielsen, Resuscitative Medicine Program, Naval Medical Research Institute

- 1:25-2:25**      **Assessment of the field in relation to the needs of the Navy**  
Panel: Dr. Oliver (chairperson), Dr. Jones, Dr. Lemasters, Dr. Proctor, CDR  
Bennett, LCDR Rhee, MAJ Verma
- 2:25-2:30**      **Close**  
Dr. Thor B. Nielsen, Resuscitative Medicine Program, Naval Medical Research  
Institute



## INTRODUCTION

Dr. Anne Murphy, George Washington University Medical Center

I'm Dr. Anne Murphy. I've spoken with most of you, or many of you, over the phone over the course of the past couple months in trying to get this meeting organized. I am a researcher like most of you, who is funded by the Office of Naval Research. I do research on the effects of hypoxia on the mechanisms of damage to cells during hypoxic periods. So I'm actually one of you, but I happen to be local and, therefore, was recruited to help organize the meeting. First of all, I'd like to introduce Thor Nielsen, who has been the major organizer of this meeting. He is with the Naval Medical Research Institute in Bethesda. I'd like to introduce, as well, two of his assistants: Johanna Kidwell and Lisa Clark-Dalton.

The next thing I need to do is introduce CAPT Thomas Contreras. He's going to introduce the general session. CAPT Contreras is a physiologist by training. He's now the Commanding Officer of the Naval Medical Research Institute. His previous position was as Executive Officer of the Naval Health Research Center in San Diego, so he brings with him not only a lot of organizational skills, but a lot of scientific background as well. CAPT Contreras.

CAPT Contreras, Naval Medical Research Institute

Thank you. Good morning and welcome to the 1996 Cell Biology of Hypoxia conference. It is a great pleasure to be your host. You've come to Washington, DC at a time when most of it's under water due to Hurricane Fran, so maybe your chances of visiting some of the landmarks around the DC area maybe limited, but take an opportunity to go down and see them. I'd like to thank each and every one of you for coming to this very important conference. We need people like yourselves to guide us along in the areas that we should focus on in the future. Specifically in this area, your help is very, very useful. The area of hypoxia is an area of research that the Navy is keenly interested in. It is very crucial to our war fighting mission. The fact that the Office of Naval Research is the sponsor of this conference clearly indicates that they also agree that this is a critical area of research. We need to do more of it. Again, I welcome you all to Washington, DC. I hope that your participation in this conference will be as beneficial to you as I know it will be to the Navy. Thank you very much.

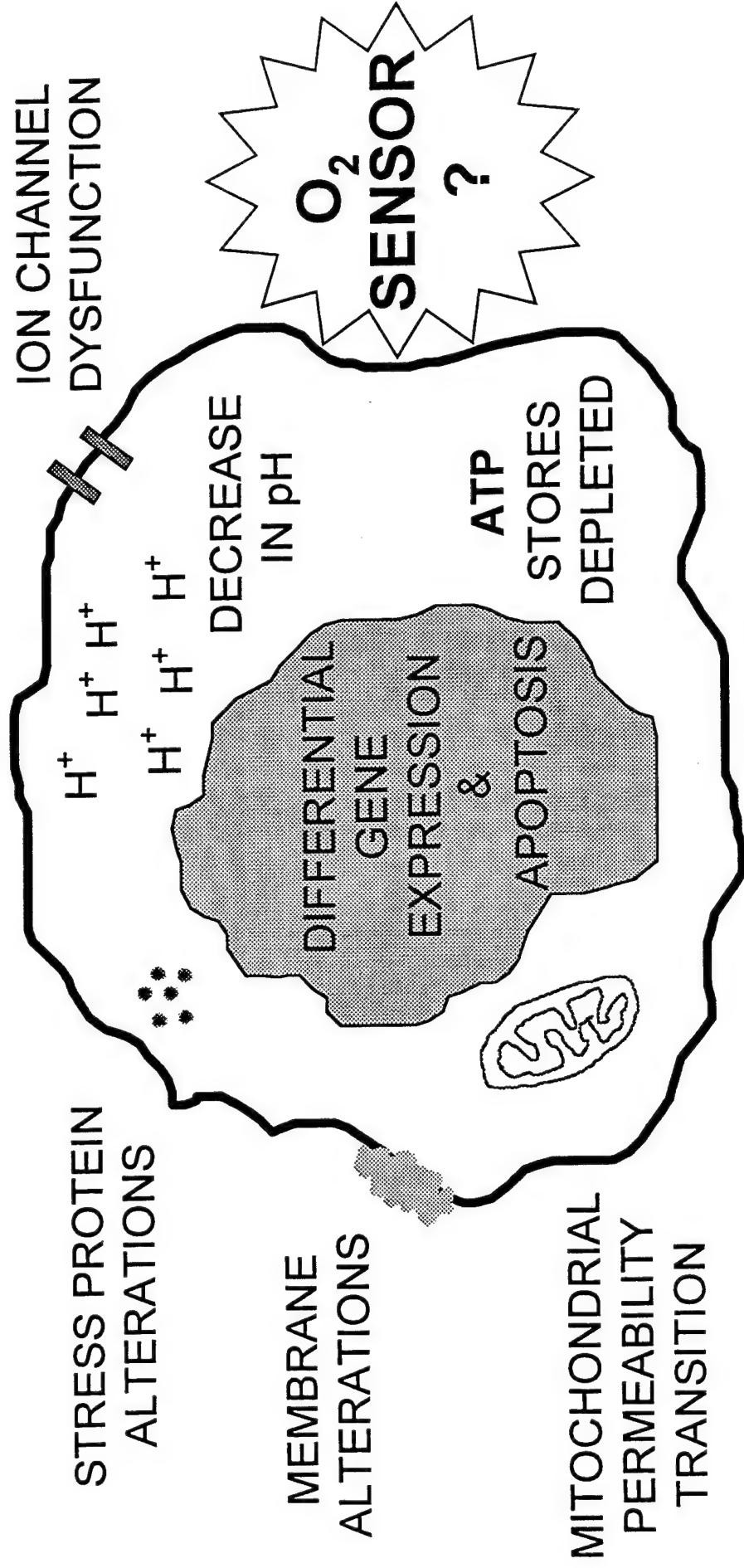
Dr. Anne Murphy

I'd like to now introduce Dr. Constance Oliver. She is in charge of biomedical science and technology at the Office of Naval Research and has been the idea person for bringing this meeting about. Many of you know her very well as the contact person of the Office of Naval Research, through which your grants have been either awarded or reviewed. She's going to give us a statement of the overall goals for this meeting and the reason that the Office of Naval Research is very interested in hypoxia. Dr. Oliver.

Dr. Constance Oliver, Office of Naval Research

Well good morning. Thank you, Anne, for your willingness to participate in this endeavor. It's a pleasure to see a lot of old friends and to have a chance to meet and talk with a number of you that so far I've only communicated with over the telephone. I want to expand a little bit on what CAPT Contreras has said, which is: why should the Navy be interested in hypoxia? To give you a little bit of a background into this program, the particular program that many of you are participants in was started in 1995. It's an outgrowth of an older program on the cell biology of trauma that was managed by Dr. Jeannine Majde, who is with us today. If we need some historical perspective, Jeannine is here to provide that as well as scientific expertise. The objective of the cell biology of hypoxia program, as I think most of you are aware, is to understand the molecular mechanisms of cellular adaption to hypoxia. The reason the Navy is interested in this particular area of research is to develop new strategies for life sustainment of combat casualties. Unlike the civilian sector that normally evacuates casualties immediately, the military does not always have this option. If you have an injured soldier or sailor on a battlefield or on shipboard who can't be evacuated, then what can we do? What mechanisms can we develop to sustain life? The basis of this program is to provide basic scientific understanding of what happens to cells when they're deprived of oxygen. Are there areas that we can exploit pharmacologically to make cells think that they're perfectly happy and in a perfectly normal environment? Without this information it will be impossible to come up with pharmacological interventions or other therapeutic strategies. This slide is a diagrammatic representation of many of the areas that will be covered in the next day and a half (Figure 1). As all of you know, hypoxia affects many if not all cellular processes. The people who are going to be speaking here today will be touching on many many different aspects of cell function including cytoskeleton membrane alterations, mitochondrial changes, ion changes within the cells, changes in gene function, gene regulation, and apoptosis. At this point we really don't know whether any or all of these areas are going to be likely targets for intervention, but the focus is to exploit what we can learn at a basic level and turn that into a therapeutic intervention. That may be to reduce cellular metabolism in such a way that cells survive the hypoxia, or to provide drugs that can treat the injury that occurs during reoxygenation. Tomorrow afternoon, the final section will be a panel discussion, and for me and for the Office of Naval Research, this is really one of the most critical portions of a meeting such as this. We've asked a number of people to participate in the panel, but we also need everyone's participation. We'd like to take that time to assess the state of the art, the state of the research. Where are we? Are there gaps? Are there areas that we need to go into? One area that I'm very interested in is identifying a cellular oxygen sensor. What we need to assess is basic research issues in a military context. Are we going in the right direction in terms of our thinking for providing pharmaceutical intervention for traumatic injury? Are there gaps in our knowledge, are there gaps in our goals, and in our targets? So I would really urge all of you, as you listen to the talks and as you give your own presentations, to think about this last hour tomorrow afternoon which is where I really would like your input and help in setting some of the future goals and the directions of the program, both scientifically and militarily. Welcome and thank you for your participation.

# *Hypoxia Affects Many Cellular Processes*



## CHARACTERIZATION OF THE MASTER REGULATOR OF OXYGEN SENSING AND UTILIZATION - THE HEME ACTIVATING PROTEIN 1

Li Zhang, Ph.D.

Department of Biochemistry  
New York University Medical Center

Heme is central to oxygen sensing and utilization in all living organisms. Both heme and hemoproteins can act as oxygen sensors and mediate cellular responses elicited by changes of oxygen concentration or hypoxia/reoxygenation in diverse organisms. Our laboratory focuses on studying the roles of heme in oxygen sensing and utilization in eukaryotic cells. Our experimental systems include both yeast and mammalian cells. We first used the yeast *Saccharomyces cerevisiae* as a model system because it is the only eukaryote in which the master regulator of oxygen sensing and utilization, the heme-activating protein 1 (HAP1), has been identified and cloned.

In yeast, heme synthesis is directly correlated with oxygen concentrations. Heme mediates cellular responses to changes of oxygen concentrations through the transcriptional activator HAP1. We found that, in the absence of heme, HAP1 is bound by certain cellular factors and forms a high molecular weight complex. This complex is critical for heme regulation of HAP1, and its formation requires the cooperation of three heme-regulatory domains of HAP1. Heme presumably binds to the heme-responsive motifs of HAP1, and might therefore change HAP1 conformation, causing the disassembly of the high molecular weight complex. As a result, HAP1 is free to dimerize and bind to DNA with high affinity, thereby activating transcription. Our studies on HAP1 will lay the basis for studying oxygen sensing in mammalian cells.

## INTEGRATION OF SENSING MECHANISMS FOR RAPID AND CHRONIC HYPOXIA

Dr. Dean P. Jones, Ph.D.

Department of Biochemistry

Emory University School of Medicine

Aerobic organisms must respond to protect against short-term interruptions in  $O_2$  supply and to acclimatize to chronic  $O_2$  deficiency. Both types of responses are essential yet serve very different functions. The former are critical to tolerate traumatic injury, cerebrovascular accidents and other ischemic conditions until  $O_2$  supply is restored while the latter are essential for an organism to function optimally at high altitudes or with pulmonary or cardiac insufficiency. The biologic strategies for these responses are opposite: tolerance to abrupt anoxia is best achieved by depression of energy requiring functions (e.g., ion transport, biosynthesis) while adjustment to chronic hypoxia involves altered expression of the molecular machinery and structural changes to optimize function. Ironically, these latter changes do not necessarily improve tolerance to anoxia but can reduce antioxidant defenses and make cells more vulnerable to ischemia/reperfusion injury. This is in direct contrast to transient exposures to ischemia which can protect tissues from subsequent ischemia "ischemic preconditioning"). The sensing mechanisms for these responses to  $O_2$  deficiency remain poorly defined, but the distinction between anoxic tolerance and hypoxic acclimatization must be recognized as studies are performed to identify these sensing mechanism. Evolution of aerobic-tolerant anaerobic microbes preceded evolution of multicellular organisms that are dependent upon  $O_2$  for energy metabolism. Consequently, primordial sensing mechanisms probably depended upon detection of either reactive oxygen species or redox changes rather than sensing of molecular  $O_2$  or cellular energetics. Existing  $O_2$  sensing mechanisms therefore probably include these parameters as well as responses to  $O_2$ , ATP/ADP, and changes in membrane potentials. For direct sensing of  $O_2$ , two general mechanisms occur, one in which  $O_2$  binding to a hemoprotein provides an initial signal and another in which the product of an  $O_2$ -dependent enzyme provides a signal. Although there are over 100 known  $O_2$ -dependent enzymes, the ones involved in heme biosynthesis are among the most interesting for  $O_2$  sensing because they are distributed between the mitochondria and cytoplasm and have  $K_m$  values that are high relative to usual cellular  $O_2$  concentrations. Thus, the concentrations of the product of the pathway (heme) and intermediates in the pathway (coproporphyrinogen, protoporphyrinogen, protoporphyrin) can vary with  $O_2$  concentration and serve in signal transduction. While the available evidence for the involvement of this pathway in  $O_2$  sensing is largely focused on transcriptional activation by heme, it is of great interest that the heme intermediates are lipophilic polyvalent anions with 5-member ring systems, physical characteristics that are shared with calciphor polymers. Di-Calciphor and tri-calciphor protect against cerebral ischemia and anoxic cell injury by protecting against mitochondrial failure. Thus, heme and heme precursors, whose concentrations vary as a function of  $O_2$  availability, may play a central role both in signaling a depression of energy metabolism to allow tolerance to abrupt anoxia as well as transcriptional regulation to allow acclimatization to chronic hypoxia.

## INSIGHTS PROVIDED BY GLYCINE CYTOPROTECTION INTO CELLULAR MECHANISMS OF HYPOXIC INJURY

Joel M. Weinberg, M.D.  
University of Michigan Medical Center  
Ann Arbor, Michigan

During the past decade, glycine has emerged as an unexpected, major determinant of cellular susceptibility to hypoxic and related forms of acute injury in diverse cell types including kidney tubules, hepatocytes, and endothelial cells. The effect of glycine is robust. Lethal plasma membrane damage that would be maximal within 15-30 min. in the absence of glycine is completely prevented for up to several hours with full effects requiring 2-5 mM concentrations of the amino acid. These levels of glycine are likely present during most forms of *in vivo* hypoxia/ischemia, but are almost always absent during *in vitro* study conditions unless replaced. Cytoprotection by glycine does not involve its metabolism, and alanine, other specific small neutral amino acids, and compounds active at ligand-gated and other chloride channels can reproduce it to various degrees. However, a binding interaction with a specific protein target remains to be defined and the plasma membrane permeability defect blocked by glycine, although having characteristics of a size-limited pore, is far larger than a chloride channel. Glycine cytoprotection does not require amelioration of any of the classical pathophysiological mediators of hypoxic injury, but, rather, allows for maximal expression of their effects before generalized, nonspecific cell disruption and, thus, provides new perspectives into likely and possible roles for a variety of processes whose relative importance has been heretofore incompletely defined despite much work. Among such events are alterations of intracellular calcium, phospholipase activation, cytoskeletal disruption, lipid peroxidation, and the mitochondrial permeability transition. Recent new insights into each of these processes from studies using glycine will be reviewed.



## BCL-2 INHIBITS CELLULAR INJURY INDUCED BY TRANSIENT CHEMICAL HYPOXIA/AGLYCEMIA AND POTENTIATES MITOCHONDRIAL $\text{Ca}^{2+}$ UPTAKE CAPACITY

Anne N. Murphy, Robert S. Balaban\*, and Gary Fiskum

Department of Biochemistry and Molecular Biology

George Washington University Medical Center

\*Lab. of Cardiac Energetics, NHLBI, NIH

Overexpression of the anti-apoptotic protein Bcl-2 inhibits the delayed death of GT1-7 hypothalamic tumor cells following transient exposure to chemical hypoxia (cyanide) and aglycemia. In addition, Bcl-2 prevents mitochondrial injury that is evident early in this death pathway (Myers, K.M., Fiskum, G., Liu, Y., Simmens, S.J., Bredesen, D.E., and Murphy, A.N., *J. Neurochem.* 65:2432-2440, 1995). The antioxidant N-acetylcysteine at 1 mM provides similar, if not enhanced, protection against mitochondrial respiratory inhibition and delayed cell death providing evidence that this early mitochondrial injury is oxidative in nature. Other experiments designed to assess the mechanism of action of Bcl-2 in prevention of ischemic damage have revealed a significant potentiation by Bcl-2 of the maximal mitochondrial  $\text{Ca}^{2+}$  uptake capacity, which is most dramatic when  $\text{NAD}^{+}$ -linked substrates are provided for oxidation (Murphy, A.N., Bredesen, D.E., Cortopassi, G., Wang, E., and Fiskum, G., *Proc. Natl. Acad. Sci.*, in press, 1996). Bcl-2 overexpression is associated with higher levels of membrane potential (as measured by  $\text{TPP}^{+}$  sequestration) and an enhanced ability to maintain membrane potential and re-reduce pyridine nucleotides following  $\text{Ca}^{2+}$ -induced oxidation. Cyclosporin A (20 micromolar), an inhibitor of the mitochondrial membrane permeability transition, potentiates the  $\text{Ca}^{2+}$  uptake capacity of mitochondria in both control cells and Bcl-2 overexpressors, and decreases the difference in maximal sequestration between the two mitochondrial types. These data support a specific protective effect of Bcl-2 on retention of normal mitochondrial function in response to high  $\text{Ca}^{2+}$  loads or oxidative stress which may provide a mechanistic rationale for the use of agents designed to protect mitochondrial function in the treatment of ischemic injury.

## PREVENTION OF pH-DEPENDENT REPERFUSION INJURY

Dr. John J. Lemasters

Department of Cell Biology and Anatomy  
University of North Carolina School of Medicine

My research interests concern cellular mechanisms underlying hypoxic and toxic injury to liver and heart cells and organs stored for transplantation surgery. In particular, my laboratory is applying new techniques of laser scanning confocal microscopy to characterize ion homeostasis, mitochondrial function, protease and phospholipase activation, stress protein expression, and lysosomal breakdown during the pathogenesis of lethal cell injury. A recent and exciting new finding is the demonstration of a "pH paradox" in ischemia/reperfusion injury. The pH paradox refers to the paradoxical worsening of cell injury when pH is returned from acidotic to normal during reperfusion. This change of pH rather than reoxygenation precipitates lethal cell injury after reperfusion. The pH paradox is likely mediated by activation of pH-dependent degradative enzymes. Significantly, lethal cell injury caused by the pH paradox can be prevented by inhibition of  $\text{Na}^+/\text{H}^+$  exchange in the plasma membrane.

We are also characterizing the role of the mitochondrial permeability transition in toxic and hypoxic injury. Increases of mitochondrial free  $\text{Ca}^{2+}$  and oxidation of mitochondrial pyridine nucleotides and glutathione promote the mitochondrial permeability transition that, in turn, leads to mitochondrial depolarization and uncoupling of oxidative phosphorylation. Recently, we showed the occurrence of the mitochondrial permeability transition in models of oxidative stress and reperfusion injury. Furthermore, inhibitors of the permeability transition, like cyclosporin A and trifluoperazine, reduce lethal cellular injury during oxidative stress and after reperfusion of ischemic cells. These findings offer new strategies to rescue cells and tissues from irreversible toxic and ischemic injury.

We are also studying reperfusion injury to livers stored for transplantation surgery. Following periods of storage associated with graft failure after transplantation, we showed that reperfusion causes sinusoidal endothelial cells to lose viability and Kupffer cells (hepatic macrophages) to become activated. Based on these findings, we developed a new solution, Carolina rinse solution, whose use during reperfusion reduces lethal endothelial injury greatly and improves graft survival dramatically. This solution is now in clinical trials. We are further pursuing mechanisms of Kupffer cell activation caused by ischemia/reperfusion, endotoxin and traumatic stress, particularly the roles played by calcium and potassium channels, endocytosis, adenosine receptors and  $\text{nF}/\kappa\text{B}$  activation.



## EXPRESSION OF HEAT SHOCK PROTEINS IN THE GUT AFTER HYPOXIA AND EXOGENOUS STRESS

Alexander K. Murashov and Debra J. Wolgemuth

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The long range goal of our experiments is to characterize the role of the hsp genes in the cellular response to hypoxic cell damage in the gut and to determine the function of the hsp in the cellular adaptation to hypoxic conditions and the development of tolerance to subsequent hypoxic assaults. We have examined the spatio-temporal pattern of expression of members of the hsp cellular stress gene family at the protein level in the gut of adult mice subjected to experimental hypoxia and heat shock. Immunocytochemical detection in histological sections showed induction of the Hsp 70 inducible family member in the mouse stomach after 2, 8 and 16 hours of hypoxia. The expression was observed in Chief cells, which secrete pepsinogen, and in Parietal cells, which secrete hydrochloric acid. Expression of Hsp32 (heat shock protein encoding Heme Oxygenase-1) was detected in the stomach at 8 hours after heat shock, in Parietal cells and in surface mucous cells of gastric pits. Expression of Hsp25 was induced by hypoxia in all regions of the mouse gut examined, including stomach, small and large intestine. In the stomach, strong induction was detected in squamous epithelium. In small intestine, the expression was restricted to Goblet cells, which secrete mucinogen and in lamina propria of villi. In lamina propria, the expression was localized to smooth muscle cells and lymphocytes. In large intestine, the expression of Hsp25 was detected in Goblet cells and Paneth cells, which secrete lysozyme. Expression of Hsp25 was also induced in circumferential and longitudinal layers of the muscularis mucosae in the stomach, small and large intestine. The results indicate that hsps are expressed in different cellular populations and in different patterns after hypoxia and heat shock. Moreover, cells with high secretory functions appeared to be particularly sensitive to oxygen depletion. These findings indicate that hsps can serve as markers of hypoxic injury in different cellular populations of the gut. We are currently generating transgenic mice to assess how gain and loss of function mutations of several hsp genes in the mouse will affect the mechanisms of cell adaptation and recovery after experimentally induced hypoxia.

## EXPRESSION OF STRESS GLYCOPROTEINS AND HEAT SHOCK PROTEINS IN RENAL PROXIMAL TUBULE CELLS AFTER TRANSIENT HYPOXIA

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The objective of this study is to characterize the regulation of J6/GP50 gene expression and glycosylation in response to renal ischemia and its relationship to expression of the major heat shock protein, HSP70 during hypoxic stress. A secondary objective is to induce the cellular stress response and upregulate J6/GP50 and other stress proteins for the development of cellular resistance to hypoxic damage. Initial efforts included the assessment of a potential therapeutic value of retinoic acid and a thiozolidine prodrug of cysteine, RibCys. Our data show that in vivo, J6 and its glycosylated form GP50 appear at 1-5 days following a renal ischemic episode of 45 min. Modeling of this response in vitro in a short-term primary culture model shows that standard culture conditions of freshly isolated proximal tubule cells is not adequate for simulating the in vivo features of the cellular stress response. A comparison of the stress response in freshly isolated tubules, in tubules grown under oxygenated conditions on plastic, or under standard culture conditions indicate the progressive capacity of the cells to respond to stress as they adapt to non-physiological conditions. This is reflected both in the accumulation of -classical- heat shock proteins and in the accumulation of stress glycoproteins. Mechanistic studies of these two components of the stress response therefore are expected to be sensitive to the details of the cell culture model. In conclusion, the ischemic/hypoxic stress response and the heat stress response involve both HSPs and stress glycoproteins. Their role in protecting the kidney against ischemic injury, and their interaction with each other remain to be defined.

## ROLE OF ENDONUCLEASE IN HYPOXIC INJURY

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I have been interested in the mechanisms of renal injury and most specifically the role of reactive oxygen metabolites in models of acute renal failure including ischemic acute renal failure. It has been generally accepted that the DNA damage induced by oxidant stress was due to the site specific generation of hydroxyl radical or other oxidant species on DNA.

Based on the ability of oxidants to increase intracellular calcium prior to any evidence of cell injury (Am J Physiol 263:F214-F221, 1992), we reasoned that endonuclease activation may play a role in the DNA damage. We demonstrated that exposing renal tubular epithelial cells (LLC-PK1) to hydrogen peroxide led to DNA fragmentation and DNA damage that was prevented by endonuclease inhibitors (J Clin Invest 90:2593-2597, 1992). This led us to consider the possibilities that endonuclease activation, classically considered to be associated with apoptosis, may be important other forms of cell death traditionally felt to result in a necrotic form of cell death.

We examined the role of endonuclease activation, considered a characteristic feature of apoptosis, in hypoxia/reoxygenation injury to rat renal proximal tubules. We demonstrated that subjecting rat renal proximal tubules to hypoxia/reoxygenation resulted in DNA strand breaks and DNA fragmentation which precedes cell death. Hypoxia/reoxygenation resulted in an increase in DNA-degrading activity with an apparent molecular mass of 15 kDa on a substrate gel. Despite unequivocal evidence of endonuclease activation, the morphologic features of apoptosis were not observed. Taken together our data provide strong evidence for a role of endonuclease activation as an early event which is entirely responsible for the DNA damage and partially responsible for the cell death that occurs during hypoxia/reoxygenation injury (Proc Natl Acad Sci USA, 92:7202-7206, 1995).

Our current studies are targeted towards understanding the mechanisms involved in the endonuclease activation. Cultured cells are very valuable for assessing mechanistic issues and have been extensively utilized to study hypoxic injury. Hypoxia resulted in an increased DNA-degrading activity with a molecular mass of approximately 15 kDa, and led to DNA strand breaks and DNA fragmentation that preceded cell death. Endonuclease inhibitors prevented DNA strand breaks, fragmentation and cell death (Kidney Intl, 49:355-361, 1996). These studies indicate the suitability of hypoxic injury to LLC-PK1 cells as model system to study the mechanisms involved in hypoxic injury.

In our recent studies, we have examined the role of reactive oxygen metabolites in hypoxic injury to LLC-PK1 cells. LLC-PK1 cells subjected to hypoxia resulted in enhanced generation of intracellular reactive oxygen species. Scavengers of reactive oxygen metabolites and metal chelators provided significant protection against hypoxia-induced DNA

strand breaks and DNA fragmentation and cell death (Am J Physiol, 271(1): F209-F215, July 1996).

Taken together, these data indicate that reactive oxygen species play a role in hypoxic injury to renal tubular epithelial cells.

## CALPAIN PROTEASES AS EFFECTOR MECHANISMS IN ANOXIC HEPATOCYTE INJURY

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Calpain proteases contribute to hepatocyte necrosis during anoxia. Our aims were to ascertain the mechanisms causing calpain activation during anoxia and to determine the mechanism by which they may precipitate cell necrosis. In rat hepatocytes, a 2-fold increase in calpain activity occurred despite the lack of an increase in cytosolic free calcium. The increase in calpain activity was not associated with an increase in calpain messenger RNA or a decrease in calpastatin messenger RNA expression. Because phospholipid degradation products generated by phospholipases can activate calpains at physiologic calcium concentrations, we determined the effect of phospholipase inhibitors and activators on calpain activity. The phospholipase inhibitor fluphenazine, decreased calpain activation and improved cell survival. Melletin, a phospholipase activator, increased calpain activity and potential cell killing. These data suggest a novel cascade for degradative hydrolase activity during hepatocyte necrosis by anoxia with phospholipase-mediated activation of calpains. The mitochondrial membrane permeability transition (MMPT) has been proposed as a mechanism of cell necrosis. Therefore, we next determined whether calpain-like protease activity may induce MMPT. A calpain protease inhibitor inhibited both calpain-like protease activity and induction of the mitochondrial membrane permeability transition by calcium. This effect of the inhibitor was specific. The protease inhibitor also delayed the onset of mitochondrial depolarization and cell necrosis during treatment of rat hepatocytes with tertbutylhydroperoxide. These data suggest a unified hypothesis linking calpain-like protease activity to the mitochondrial membrane permeability transition in cell necrosis. We propose that in this degradative hydrolase cascade with phospholipase mediating activation of calpains that mitochondria are the target of calpain activity which then leads to cell necrosis.

## THE ROLE OF BCL-2 HOMOLOGUES IN HYPOXIA AND GROWTH FACTOR STARVATION INDUCED APOPTOSIS

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Endothelial cells are uniquely positioned to encounter a variety of substances that might cause injury to these cells, including humoral factors, mechanical factors and inflammatory molecules. However, these cells have a very long lifetime with turnover rates being months to years. Thus, cell death must be a rare event for endothelial cells suggesting that these cells are protected from programmed cell death (apoptosis). We have examined cell death resulting from apoptosis induced by growth factor starvation and hypoxia. Human umbilical vein endothelial cells (HUVEC) in separate experiments were subjected to a hypoxic environment or growth factor withdrawal to induce apoptosis. We have shown that hypoxia requires approximately 48 hours to induce apoptosis leading to the conclusion that these cells are resistant to hypoxia relative to other cells types. Northern blots of RNA from hypoxic cells at 24 and 36 hours had increased expression of the Bcl-2 homologue A1 and the cytoprotective molecule A20. Expression of mRNA at 48 hours for these molecules has decreased back toward baseline. These data are consistent with A1 and/or A20 being cytoprotective soon after initiating hypoxia. Addition of basic fibroblast growth factor (bFGF) to starvation medium prevents apoptosis and cell death beginning as early as 3 hours after addition of bFGF. Western blot analysis shows an increased expression of the cytoprotective molecule Bcl-2 at 6-9 hours. There was no increased expression of other known members of the Bcl-2 family. These data are consistent with Bcl-2 providing late protection in HUVEC but do not explain the early protection.

## MECHANISMS OF DEATH IN CEREBRAL ENDOTHELIAL CELLS IN RESPONSE TO HYPOXIA, HYPEROXIA, OR INFLAMMATORY SIGNALS

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Cerebral endothelial cells (CECs) are the interface between blood/blood cells and the brain. CECs die by necrosis or apoptosis under different experimental conditions. CECs are the major site of disturbance in a number of pathological states including hypoxia, hyperoxia, or inflammatory/infectious disorders which are frequently encountered by Navy personnel. We explored mechanisms of CEC death in response to hypoxic or hyperoxic condition or inflammatory signals. Apoptosis can be induced in CECs by exposure to hyperoxia or following sequential treatments with tumor necrosis factor alpha (TNF $\alpha$ ) and cycloheximide (CHX). CEC death triggered by hyperoxia or TNF $\alpha$ /CHX shared a number of common features of oxidative stress: DNA fragmentation and laddering, positive TUNEL stain, and an increase in NF-kB binding activity. DNA fragmentation in CECs induced by hyperoxia can be inhibited by N-acetyl-cysteine, melatonin, or a novel antioxidant, carboxy-buckminsterfullerene. CEC death induced by hypoxia is primarily a necrotic process which is characterized by early membrane breakdown and release of LDH in contrast to apoptosis triggered by hyperoxia and TNF $\alpha$ /CHX which is associated with early DNA fragmentation in the absence of LDH release. Understanding the differences in mechanism of CEC death under various pathophysiological conditions may aid in the future development of specific therapeutic strategies directed at particular disease states which are more commonly encountered by Navy personnel. (Supported by an Office of Naval Research Grant: N00014-95-1-582-01)

## ORALLY ADMINISTERED IL-6 AS PROPHYLAXIS FOR SEPSIS FOLLOWING HEMORRHAGIC SHOCK

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Orally administered IL-6 has been shown to reduce bacterial translocation from the intestines of hemorrhaged mice and rats. The mechanism(s) for such beneficial effects have not been elucidated. We investigated the ability of oral IL-6 to affect microcirculation in the ileum following hemorrhage. Doppler flow measurements, as well as E.M. studies using iv administered HRP, showed that blood circulation was markedly reduced following hemorrhage with resuscitation. Following IL-6 administration, however, the intestinal microcirculation was again patent as demonstrated by increased HRP permeability and Doppler flow. Intraluminal HRP was shown to pass between intestinal epithelial cells of hemorrhaged mice, but not in hemorrhaged mice fed IL-6 or normal mice. Since the IL-6 effect on intestinal microcirculation occurs within 3-5 minutes, as measured by Doppler flow, it is unlikely that enzymatic digestion of IL-6, which reaches maximum in 30 minutes *in vitro*, can completely abrogate the effect. We propose that a mechanism of action of IL-6 in intestinal ischemia is to relax the intestinal microvasculature, thereby allowing restored oxygenation of tissues.



## TRAUMA AND HEMORRHAGE ALTER *IN VIVO* CELL PROLIFERATION, GENE EXPRESSION, AND APOPTOSIS

Dr. Thor B. Nielsen

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The form of ischemia of most direct military significance is hemorrhage. Hemorrhage is frequently associated with trauma and complicates the course of recovery and treatment. Work in other laboratories has defined the major physiological consequences of hemorrhage and important work has been done on cellular function during trauma and hemorrhage, including regulation of vascular tone and activation of cell function. Resuscitation with acellular oxygen carrier solutions offers the potential advantage of improved oxygen delivery compared to crystalloid solutions, but the cellular consequences of improved resuscitation have not been fully evaluated. The status of cell death, cell proliferation, stress protein response, and early gene expression in a living tissue or organ is pivotal in understanding the pathophysiology of trauma. Our studies evaluated local and systemic cellular effects of trauma, hemorrhage, and resuscitation in a model of combined hemorrhage and surgical trauma. *Methods:* Trauma was modeled in rats by full-thickness shoulder to pelvis mid-line incisional wounds and hemorrhage by fixed volume depletion. The *c-fos* mRNA was determined by non-radioactive *in situ* hybridization with a biotinylated rat *c-fos* oligonucleotide probe. HSP 70 was investigated by immunohistochemistry using a monoclonal antibody against HSP 72/73. Cellular proliferative responses were evaluated by labeling *in vivo* with 5-bromo-2'-deoxyuridine. Apoptosis was characterized immunohistochemically by TdT-mediated dUTP nick end labeling (TUNEL). Diaspirin crosslinked hemoglobin (DCLHb™) or shed blood were compared for resuscitation. *Results:* The *c-fos* mRNA was detected in the epidermis soon after trauma and peaked at 6 h post-injury. DCLHb resuscitation modified the *c-fos* mRNA expression in epidermal keratinocytes. HSP 70 was rapidly expressed in epidermis and hemorrhage enhanced expression. Trauma inhibited keratinocyte and hepatocyte proliferation soon after the trauma, and stimulated subsequent proliferation of keratinocytes and liver non-parenchymal cells. DCLHb stimulated wound keratinocyte proliferation and attenuated the inhibition of hepatocyte proliferation. The skin cells most active in both proliferation and apoptosis appeared to be keratinocytes. Normal rat skin had low rates of proliferation and apoptosis. The ratio of proliferating to apoptotic cells (P/A), an indication of deviation from homeostasis, was strongly decreased in response to hemorrhage and closer to normal after administration of DCLHb, suggesting improved cell vitality. *Conclusion:* Trauma alone, or in combination with hemorrhage, modulated cell proliferation both in the wound and in the remote organs of intestine and liver. DCLHb enhanced wound healing and cell proliferation as well as, or better than, freshly-drawn blood, and may be beneficial for trauma care. The co-expression of *c-fos* gene and HSP 70 can be used as an indicator of pathophysiological response to hemorrhage and acute wound. The *c-fos* gene and HSP 70 expression in epidermis may be a sign of traumatic severity whereas *c-fos* induction and inducible protein HSP 70 in sebaceous glands may reflect the strength for protection. If so, DCLHb seemed

to be most effective for resuscitation in this study. These data suggest that resuscitation with blood or DCLHb is important not only for the survival of the organism, but also for skin remodeling and keratinocyte viability.

## DEVELOPMENT OF CLINICALLY-RELEVANT MODELS OF TRAUMATIC SHOCK

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Despite overwhelming evidence favoring various anti-sepsis strategies in numerous experimental animal models, virtually all the large scale, multi-center trials have yielded disappointing results. Costs have exceeded tens of millions of dollars. The profound disparity between the experimental and clinical results can be attributed to one of two simple explanations: either animals react differently than humans to the same stimulus, or the experimental models do not accurately reflect the real life situation.

Consider the following: First, otherwise healthy humans do not spontaneously develop sepsis, but a typical experiment uses healthy, drug-free animals on fixed, standard diets exposed to extraordinary septic challenges in the absence of even the most basic standards of care (i.e., supplemental oxygen, fluid, ventilatory support). The pathophysiologic responses in such extreme conditions are rarely seen in real life, so it follows that therapies that are effective in those models might not necessarily apply to humans. Second, precise timing of the therapeutic intervention is critical for influencing outcome. The same treatment applied early can have opposite actions if applied late. Unlike the models, the exact time course may be difficult or impossible to track in most septic patients, so it follows that those therapies will have variable results. Third, most septic patients, unlike the experimental animals, will eventually recover, even though mortality is often the main, albeit crude, endpoint in both and clinical and experimental trials.

To address some of these problems, we have narrowed our research focus to the causes and treatment(s) of sepsis following trauma. The trauma patient, like the experimental animal, is usually healthy at the time of injury. In our models and in trauma patients, the priming insult and the therapeutic intervention can be precisely timed. Major advantages of the model are that multiple invasive variables can be monitored and the magnitude and location of the injury can be controlled. After traumatic shock (hemorrhage combined with various tissue injuries), pigs are resuscitated with protocols similar to those in most urban trauma centers. Not surprisingly, the results are variable, but remarkably similar to those in patients. We have demonstrated trauma-induced changes in pulmonary function and the efficacy of therapeutic interventions that replete high energy phosphates and which target leukocyte-mediated reperfusion injury. We have evaluated effects of alcohol or cocaine because a large fraction of trauma patients are intoxicated. We have shown the relative risk of transfusion for increasing post trauma sepsis. These results provide a firm foundation for well-designed future clinical trials of novel therapeutic strategies. In addition, these data provide insight for the development of new models that more faithfully mimic civilian or military trauma and the complications of post trauma sepsis.

## PANEL DISCUSSION

Dr. Thor B. Nielsen, Naval Medical Research Institute

We've heard rather extensive discussions of cell biology and we've heard some work on animal models of trauma and resuscitation. I'd like to take two minutes and introduce a different topic, but one in which I know many of you have an intense interest. In this slide are the national costs of several major categories of disease (Figure 2). They are shown in red (cross-hatched bars, right axis) in billions of dollars, and the categories of diseases are cardiovascular diseases (labeled heart); the traumatic diseases, injuries, and accidents (labeled trauma); arthritis and musculo-skeletal diseases; diabetes; cancer; and AIDS. These data are from a study by J. Charnow from this year<sup>1</sup> and from work by Jaffin, et al. from a couple years ago<sup>2</sup>. These are broad categories of disease. These are *estimates* of the costs in the United States for treating the population that is afflicted with heart disease, trauma, and these other diseases, in billions of dollars. On the same graph (left axis, solid bars) is plotted the National Institutes of Health (NIH) budget for fiscal year 95 which is applied to research in these same broad disease categories: heart disease, trauma (including burns, general trauma, and trauma to head, CNS, and spine), arthritis, diabetes, cancer, and AIDS<sup>3</sup>. The NIH spends, by far, the most of any government agency on national health research. You know that the Department of Defense (DOD) also has a budget for biomedical research. It is small by comparison, but it is of interest to know that most of those funds are also spent on cancer and infectious diseases, so if you add those figures in, the overall distribution does not change very much. In times of war, casualties are likely to have mostly problems of trauma and infectious diseases of various kinds. You can see from this graph is that there does not seem to be a very close relationship between the costs of a disease and the amount expended on research to alleviate it. I hope that the panel can keep this kind of information in mind as they consider research needs for the Navy in the related fields of hypoxia, ischemia, and trauma.

The panel members are: from my left, LCDR Peter Rhee who is a *bona fide* trauma surgeon; MAJ Verma from the Uniformed Services University of the Health Sciences (USUHS), a neurologist; you've already met Dr. Jones, further introduction would be pointless; Dr. Oliver will be chairing the session, she is sitting in the middle and is easy to distinguish because she is wearing red; CDR Bennett is also from USUHS, and has been very much involved in the deployment of troops; Dr. Lemasters, you've met; and Dr. Proctor is seated on the end. So I'll turn it over to you, Madam Chairman.

Dr. Constance Oliver, Office of Naval Research

What I'm asking the panel to do, and what I'd like to accomplish in the next hour, is to have CDR Bennett and LCDR Rhee 1) give us their perspective from the Navy point of

<sup>1</sup> J. Charnow. *Infectious Disease News* 9:1, 1996.

<sup>2</sup> J.H. Jaffin, H.R. Champion, B.R. Boulanger. *Economic Considerations. Crit Care Clin* 9:765-774, 1993.

<sup>3</sup> D. Ralbovsky (NIH) and Norm Oliver (NIH), personal communication.

view, and from a trauma surgeon's point of view, of some of the realities of military research; 2) put the basic research that most of you are engaged in into a broader perspective; and 3) give us some of their impressions of where there are holes and gaps, that research could be beneficial. I've also asked MAJ Verma to do the same thing from the perspective of the Army. Following that, I'd like to turn our attention to the meeting itself. I've asked the remaining members of the panel to discuss where there may be research opportunities within the overall program that was presented today and yesterday. Are there areas that should be explored further? Are there topics that weren't mentioned at all that people think are important? We have had some discussion among ourselves about model systems. How reproducible and how translatable are model systems from one investigator and one laboratory to the other? With that introduction, I'll turn things over to CDR Bennett and ask him give you a broader perspective of the problems encountered in military medicine.

### CDR Brad Bennett, Uniformed Services University of the Health Sciences

Thank you, Dr. Oliver. I'd like to thank Dr. Nielsen for initially making contact with me as well as Dr. Oliver to elucidate what are the expectations of the panel. I'd also like to thank Dr. Jones, Dr. Proctor, and Dr. Lemasters for chairing the sessions and making their presentations, and particularly to you, as researchers in the academic community, for showing the interest to draft a proposal, submit it to the Office of Naval Research, subject yourself to critique from a peer review, and being selected to make a difference for the sons and daughters of Americans: our sailors, our marines, our soldiers, and airmen. I'm in the Department of Military Emergency Medicine at USUHS in Bethesda, and our focus there is certainly to train quality medical students. However, there is a large thrust in basic research and providing the Masters degree in Public Health and graduate degrees in other departments. But my concern is focusing in on the global thrust of why we're doing this research. Dr. Proctor also presented some slides earlier today on some of the Viet Nam War data. Where are we going in the sense of war fighting strategy? Therefore, how should our health service support component of each service then support that line command, those assigned to the ships, those ground pounders or ground forces, the Marines, the Army, and also the Air Force. Our war fighting strategy is changing rapidly as we speak, and I've been fortunate in the last two months to have been in a couple high level meetings associated with that war fighting strategy. Then how are the medical departments of each service going to support the war fighting? I think you need to understand that a lot of the models, the casualty prediction models that have been used in the Desert Storm War, are really data of the past: data of Viet Nam, data of Korea, of World War II, and World War I. The Department Of Defense and all of the commanders and chiefs that are in charge of all the unified commands, like General Schwartzkopf, who you may remember very well I'm sure from Desert Storm, depend a lot on this input to get the civilian laboratories, as well as the Department Of Defense laboratories, to develop and come up with casualty prediction models based again on data in the past. When was the last time we fought a war the same way previously? The answer is were not. War is changing. War of attrition will not be the way we fight wars in the year 2000. So we'll be fighting wars very quickly, very rapidly, with overwhelming forces. Even to say the types of injuries that you see, and 98% of the injuries that occurred in Viet Nam,

are from the operational ground forces. So the thrust of research certainly should stay within that focus for the ground forces. When we talk about Navy, for some of you who aren't familiar with the military institution, we're talking about the Navy Department and that includes US Marine Corps as well as the sailors that are at sea primarily. And we have seen some data earlier today, as well as earlier in the week, about the magnitude of injuries that occur. From a retrospective analysis of those data, at war time, casualties of sailors at sea have almost 57% mortality from catastrophic accidents [as well as 50% of these are injuries]. We talked about the Stark incident earlier today and that the Exocet missile that was launched at the USS Stark from the Iraqis killed 37 people outright and injured an equivalent number. So the profile and the types of injuries we see at sea are different than we see for the ground forces. I think that one of the slides elucidated the types of injuries; blast injury, crush and traumatic injuries, burns and inhalation injuries. There are all types of other injuries that go on on a daily basis that are pretty much managed by prevention programs. Our concern is really not prevention programs. The focus of this orientation the last two days, rightly so, is casualties. There is a tremendous emphasis in military medicine to focus in on what effect the majority of the ground pounders, the Marines and Army soldiers, have to do with non-battle disease injury. That means every type of illness or injury that occurs not in relationship to mortality and morbidity associated with fighting the war. There is a tremendous amount of emphasis on infectious disease control, malaria control, and providing prophylactic treatment, and that's a whole other thrust that takes a lot of energy and a lot of research that occurs in your institutions as well as the Department of Defense laboratories. But for those injuries that occur from battle injury itself, there is a tremendous amount of focus that should be maintained on the hemorrhagic model and a tremendous focus that should be maintained on septic models. I'm not going to address the types of models you can use, but I do want to state for those who are just getting into this type of research, what we call the 6.1 level of funding, of the basic research are not be discouraged. Because in an analogy of a book salesman going door to door, you have to go through so many "no"s to get to the "yes", meaning the guy who buys the book. From a basic research approach, we have to maintain an appropriate level of funding and focus on basic research with ultimately high risk high dollar obligations with potentially a high risk payoff down the road. If you don't see a transition in the type of research you're doing, whether it's at the cellular level or the sustainment of shock type model and *in vivo* and *in vitro*, it really doesn't matter to me. Ultimately, we need to have a transition of the work that goes from the basic research ultimately to a product for the end user, that being the medic or corpsman or the 2nd or 3rd or 4th echelons of care. That meaning someone who does life sustaining intervention, maybe an emergency room physician, a doctor or a physician assistant who gets Med-Evaced, as we call it, evacuated to a mobile hospital that's set up in the theater and then ultimately maybe to a hospital ship that basically is a floating hospital, that'll do every type of intervention necessary to sustain a life. But the hospital ship will not do the types of surgery that will be of a reconstructive type. That'll be back in CONUS (Continental United States), Walter Reed and Bethesda Naval Hospital are examples of where reconstructive type surgery will go on 30 and 60 days, and it could be even longer, after injury. So the focus, I think, really still needs to be at the forward edge of the battle area: sustainment of life, maintaining critical life saving intervention. So hemorrhagic models certainly need to be there. Emphasis on trauma



soft tissue injury models needs to be maintained in the basic research. Bellamy, a physician that is due to retire if he hasn't retired already, is a Colonel in the army and he's in the area here. COL Bellamy is an adjunct professor in my department as well as in surgery with Dr. Rhee. He's established and he's a tremendous asset to have in the area and he'll continue to be a tremendous asset. But 50% of the US injuries in the Viet Nam War were soft tissue injuries with minimal to moderate severity that were not life threatening. Also there was a tremendous amount of exsanguination, people that died because of tremendous hemorrhaging on the battlefield. Fifty to sixty percent of the injuries which occurred during the Viet Nam conflict were to the extremities; traumatic injury to the extremity caused by missile fragmentation. The majority of the injuries, those who were wounded in battle and those who got to a treatment facility, really occurred from missile fragmentations. Now we mostly think of small arms fire, that being a semi-automatic, an automatic rifle, or a nine millimeter caliber high velocity pistol, as being the cause of injuries to most of our ground pounders in Viet Nam. The majority of those who die, die from small arms fire. Yet a large percentage of injuries occur from fragmentation, and we think of a single type injury typically in these individuals but in reality they are multiple type injuries. They have multiple fragmentation so they may have multiple sites that they are bleeding from. The corpsman that works for the Marines and the medic that works for the Army are trained to deal with exsanguination. They know from early onset of their basic medical training that they need to deal with the hemorrhagic side of that casualty. So they may go up to that battle casualty in the line of fire and deal with that with a tourniquet. So a tourniquet model of research in dealing with hypoxia and anoxia in extremities certainly should continue to be a research focus. So this clamping, if you will, of a major vessel, then a reperfusion once he gets to a treatment facility, is going to occur. Perfusion injury research, because of that scenario, needs to continue and I challenge you to continue those lines of investigation. There is another area I just want to briefly touch on because we've talked about hypoxia induced mechanically, in this case say a tourniquet, or chemically. You've given us some sophisticated models of hypoxia in the last couple days and I applaud you for using well balanced and reproducible models. You may not see the application to the clinical relevant model at this point, but that's okay. The transition that's done in research is, as I call it, really going to be the responsibility of Dr. Oliver and her colleagues at the Office of Naval Research: to look to the next step of funding, ultimately coming up with a final product for the end user. But something that wasn't touched on, and maybe rightly so, are concerns of weapons of mass destruction. Those weapons of mass destruction are radiation syndromes from nuclear weapons, biological infections from anthrax, botulinum toxin, plague, so on and so forth, and many others, as well as the chemical injuries that are induced. Sarin, Soman, VX and Tabun are your classic nerve agents that are used by 3rd world countries, and agents all induce rapid death. The LD50 (the lethal dose of 50% of the population) for nerve agents is 10 micrograms, that's just a dot of fluid on a penny: very, very small. These agents will cause rapid cell death in the bone marrow, and so we have already talked about cytokines and cytokine therapy. Cytokine therapy for a nerve agent patient as well as the radiation syndrome patient (lowest level being hematopoietic syndrome) in which cytokine therapy stimulates stem cells is a very important type of research. So I challenge you to not only look at a univariate model in your research, but a multivariate model just as the Armed Forces Radiobiology Research Institute right here in

Bethesda is now looking at the synergistic effect of acute radiation syndromes all the way from the low level hematopoietic effects to the CNS model, which is around 900 centigray of radiation and induces death very rapidly through a cascade of effects. But they're looking at the synergistic effects of a biological weapon, as well as radiation syndrome, both suppressing immunological functions. If a traumatic injury occurred first and then the radiation or, conversely, the radiation first and then the traumatic injury, the response and the mortality of those types of injuries are completely the opposite. I also challenge you to take a look into collaborative efforts looking at injuries as you showed in some of your cellular models. If you are exposed to a pulmonary toxic agent, e.g., phosgene, and blood toxins such as cyanide, again we're inducing hypoxia by weapons of mass destruction. When chemical agents are combined with hemorrhage and trauma, are the physiological cascade of events the same or are they different? So we need to broaden our perspective to look at synergistic injuries with weapons of mass destruction. I think with that note I'm going to stop, Dr. Oliver, and let someone else take over.

Dr. Constance Oliver

Thank you very much, I'm going to change tactics a little bit. I'd like to have Dr. Rhee give us his impression of opportunities and problems from the perspective of a trauma surgeon who would be ultimately, medically, the end user of any products that we hope to develop from this program.

LCDR Peter Rhee, Uniformed Services University of the Health Sciences

Thank you, Dr. Oliver. First I'd like to say, Dr. Oliver and Dr. Nielsen, that this is a very high quality meeting and I've really enjoyed the presentations. I'd also like to applaud all the people who have presented their work. What I'd like to talk about today is basically who I represent and what I, as a commissioned officer, would like to see come out of these types of meetings and research. Keeping in mind that I also do research myself so I have my own inherent biases, I'd like to say that I represent not just the Navy but coming from the Department of Surgery at USUHS, but I think of myself as more tri-service. I think that just like Dr. Oliver stated, even though this is Office of Naval Research and the Navy research funds, they're the only ones that fund combat casualty care in the military. So I first want to say that when I think about who my patients are going to be, it's not going to be just the Navy personnel on the ship, but I think most of the time it's going to be the Marines who are getting shot and blown up. It is also going to be the Army and Air Force people as we get more and more tri-service, along with the prisoners of war. I know that when I'm out there in the fields and doing trauma surgery those are the types of populations I can expect. The type of injuries that I can expect are people who are basically shot or blown up or burned and I don't have to worry about too much of the other things. And since we're doing research in combat casualty care, that's basically just trauma research and trauma care. So when I think about that I like to always go back on the data that is available. You know, when you think about four or five different scenarios in the future as to what the military scenarios are going to be, that's really difficult to say. But since I do fundamentally just think about people who are shot



and blown up, when it comes to it, the data that is available and is still relevant to a certain degree, is from the Viet Nam data that we have from Dr. Bellamy. This data shows you that one of the major contributors of death and one of the most important things that we think about is hemorrhagic shock. I think about everything back to that area of research and say "how does this apply to hemorrhagic shock to protect us". So I always think about hemorrhagic shock as the fundamental problem that I need to see and care about. So I always think about how is this going to affect my therapy, or is this going to change my practice? I mean, from the Korean War we learned how to give blood to save lives and from the Viet Nam war we learned how to give crystalloids and prevent renal failure and at the same time delaying death. So if you look at where the majority of the deaths on the field occurred, they did occur on the field, and they occurred from hemorrhagic shock. So when I hear about things such as hypoxia, I ask myself "do my patients get hypoxic and how important is the mechanism of hypoxia in my patients". So I think that one of the most important things when we study hypoxia, for example, is to be always able to keep an eye on the bigger picture itself and show me how this is relevant to me. If I hear that some things are just reproducible in very unrealistic environments then I don't really know if it would be applicable for me as a trauma surgeon who also does critical care on patients afterwards. I just want to say that I think it's really important to keep the clinical picture in mind. Also, knowing that research has begun in all ranges, meaning down at the cellular level and all the way up to the clinical level and one can't be done without the use of the other, so that has to be all married together. I think that's probably the biggest key. I'd like to say that if we can all, instead of just being segregated by ourselves, use collaborative efforts both as clinicians and also basic scientists, to keep an overall picture of where we're going with this, and ask ourselves "is this data going to be of any use or is it going to have any relevance to the future"? And that's all I really have to say.

### Dr. Constance Oliver

The next person that I'd like to hear from is MAJ Verma, his perspective both as a researcher and as a representative of the army.

### MAJ Ajay Verma, Uniformed Services University of the Health Sciences

I might add that I'm also a clinician so I agree with some of the things my colleague said. The brain suffers hypoxia not only in hemorrhagic injury. The head is a closed vault, so any kind of space-occupying lesion in the brain, whether its a blood clot or any kind of trauma, diminishes blood flow and results in significant hypoxia to the brain. Hypoxia is a big issue in neurology and I applaud all the work that's being done in this area. From the Army's perspective, I think the interest is in very far forward research. They are not big fans of basic research and want research that can be applied immediately to help the soldier that's down and out in the battlefield. They're interested in trying to get findings quickly from the bench to the clinic or to the field application. In that regard, I think there are certain areas of research that are more exciting than others. Some research tends to effect changes in the paradigms in the way we actually treat patients. One area that comes to mind in brain injury for example,

is the study of secondary cascades of injuries that happen following the initial injury. That gives us an opportunity to intervene, and I think that's changed the view of a lot of clinicians as far as what they can do for brain injury. There are other paradigms that we've heard about, I think, at this meeting. For example, instead of focusing on calcium overloads perhaps we should focus more specifically on the mitochondrial permeability transient. The concepts of delayed cell death and programmed cell death, I think, are very important to appreciate in terms of really getting a handle on how we should approach treatment of a patient. Research that focuses on transition between basic research and clinical applicability should also be applauded. We need to have some clinical markers that tell us which animal research models are applicable to the clinical situation. Any kind of research that can be done to link the bench top to the clinical arena should, I think, deserve a lot of focus. The other thing is that there are many targets that have been identified, as targets of intervention in preventing hypoxic cell injury. Several of these targets, I think, could be attacked with drugs that are already available clinically. There's a lot of effort sometimes spent on developing new drugs, but there are several old drugs that we're well familiar with as clinicians (we know how to use them, we know the toxicities, and so on) that could possibly be used in the same arena. I think we ought to focus a little more on that because that will help us get the product to the market much faster. Well, I think I'll stop there because a lot of the things that I wanted to say have already been said by my colleagues.

Dr. Constance Oliver

What I'd like to do now is to take any questions to myself or to the panel specifically on the military relevance of this program that any members of the audience may have.

Dr. Sudhir V. Shah, University of Arkansas

One thought, in reference to hemorrhagic shock and sepsis. I've heard reference to soft tissue injury but I've heard very little about muscle injury, *per se*. I would have thought that would be of major interest to the Armed Forces. Am I misunderstanding you or is that not a major interest?

LCDR Rhee

Well, the correct therapy for limb injury is just direct pressure. So as Dr. Bennett talks about, we're not going to have all out fights like we used to have. Rather there are going to be control type actions. So certainly the recommendations of how we treat limb injuries are different. And, for example, when we have special forces that go out, although the current therapy is not to put tourniquets on, tourniquets may become a reality in the future and be useful, especially when we think about lives being saved at the expense of a limb. So for muscle, *per se*, it's usually epitonic injury and then there's usually another secondary injury associated with it. Soft tissue injury has not caused much morbidity in the past. So I'm in agreement that it's not much of an issue. As far as the mechanisms of sepsis or hemorrhagic shock, I'd like to take this one second to say that I think there's a very distinct difference

between exsanguination, hemorrhagic shock, anemia, hypoxia, and ischemia. So when we talk about hypoxia from total occlusion and reperfusion, for example in transplant therapy or areas such as stroke, it's different than hemorrhagic shock where we don't really have full occlusion. For me as a clinician and critical care specialist, I don't really worry about true hypoxia because it's very difficult to get hypoxia at the tissue level after hemorrhagic shock or during hemorrhagic shock itself.

## Dr. Constance Oliver

Any other questions? What I'd like to do at this point is take this opportunity to get an assessment, both from the panel and from the members of the audience, of where we are within the field scientifically given the caveats of where we would like to ultimately go with a clinically relevant product. Also, I would like to ask the members of the panel and the audience that, whatever their particular model system, they control the urge to say *that's* the most important thing that's being done scientifically and *that's* where all the emphasis should be placed. I'd like to try and get an objective feeling from everybody including the members of the panel in terms of needs and stats and caveats. I'm going to start with the members of the panel. I think you're all coming from different perspectives, John, Dean, and Ken.

## Dr. Ken Proctor, University of Tennessee

One point I have is: before we begin evaluating something, don't we have to identify the problem? You state that the battle of the future is going to be different than the battles of the past, but the models we're working on are based on the statistics of the past. Do you think there is a problem with those statistics?

## CDR Bennett

Yes, let me just enlighten you a little bit more about some of the war fighting strategy changes as they're going to be fought in the year 2000 and beyond. The Marine Corps is currently working on war fighting strategy for 2010. If our line counterparts, those who are trigger-pullers (for example, aviators, submariners, tankers, etc.), if they're changing their war fighting strategy then the health service support component of each service also has to follow along in parallel. And so you may have heard in the media that the blue water Navy now has moved into the littoral, the shallow water for lack of a better term, and changing some of their service platforms to get more shallow, to bring the forces in closer. A lot of the hardware development is to support that new war fighting strategy. Analogous to that concept is what happened just recently in Iraq. Why send in the most critical assets that the Department of Defense has, that means the warm-blooded sailor, soldier, airman, our sons and daughters, so to speak? Why not use stand-off technology? Certainly that is the best way to fight a conflict. However, sometimes, as we know, we do have to put a body in the field, to go in and meet our objective. So the concept of fighting a conflict today is going to be very rapid and very mobile. So the health service support and all the other logistics support of the battle has to be able to keep up. The magnitude of injuries that we're going to see are

going to be lower certainly. The casualty prediction models that have been used over the years are based on previous war time data so they are not necessarily going to reflect the new war fighting strategy. But the types of injuries, and that's the bottom line, that we're going to see are still going to be the same types. You're still going to have a hemorrhage injury on the battle ground. You're still going to have massive trauma. You're still going to have muscle injury, but you're not going to see the muscle injury or the crushing injury on the battlefield, but on ship where the industrial type injury does occur. Again we're talking about percentages of injuries, so should muscle injury be a major thrust? I'd have to say it's probably not a major thrust, but the types of focus that you have now (sepsis, hemorrhage, and hypoxia models), will be relevant for the year 2000 and beyond.

## Dr. Ken Proctor

A follow up question concerns the application of this technology to civilian use. For example, since we don't have the Cold War anymore, we're liable to have use of the military for other disasters, such as Hurricane Fran. If the military helps to care for civilian injuries, it seems that a lot of this technology might apply to the types of injuries that would be seen in the urban trauma center. I wonder if you could comment on that? These are not planted questions by the way.

## CDR Bennett

The term "dual use" really came foremost in the Department of Defense under the current administration. Some of the thrust and focus of research dollars to support dual use started at that time. Yes, there are tremendous amounts of technology transfer that can occur in the Department Of Defense and be utilized in urban setting. It is relevant. I just want to point out for some of you who aren't familiar with some of the data by Bellamy and others, this Journal here is Military Medicine. It might be a particular journal that you might find to use to set up your Introduction, set up your review of the literature, that may put a military twist to your future proposals and give them the right flavor. In this particular journal you'll see articles by Bellamy, Carey, Dunnigan, and others who write on combat casualty care. This particular supplement I have right here, I brought in for Dr. Rhee based on talking to him yesterday. The military is moving very rapidly in fluid resuscitation on the battlefield for use by the forward advanced corpsman or medic. This particular thrust has to do with the special forces medic, those who support Navy seals and the Green Berets and those types of individuals. They have already set in here an algorithm<sup>4</sup>, if you will, based on the ABCs, and fluid resuscitation being part of the ABCs, on when they conduct stages of care, e.g. care under fire, tactical field care (meaning you're out of the direct line of fire but you're still in a tactical environment), and then care under a Med-Evac or evacuation from the battlefield. There are three sets of algorithms, so this should give you some focus. In this particular thrust with the Special Forces community, a tourniquet is a primary focus for managing hemorrhagic shock.

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<sup>4</sup> Butler FK, Hagmann J, Butler EG: Tactical Combat Casualty Care in Special Operations. Military Medicine 161, Suppl 1:3-16, 1996.

Dr. Dean Jones, Emory University

I'll just make a comment. Concerning the issue of acute anoxic injury, that is, the immediate failure of cell function, it's really one of the questions here that is of most relevance. If you can't get the cells or organs to survive long enough to get to prevent irreversible loss of function. One of the things that came out clearly in the talks is that there really are variables. The work like Dr. Weinberg presented and the solutions such as John Lemasters is working on suggest there are a variety of ways that one can manipulate how fast cells die. What we've looked for over the past twenty to thirty years is some miracle compound that we could add and would provide that additional time to get supportive treatment. I think it's out there. I think that we just haven't found it, and one of the problems has been in the movement of science. Science goes through fads and we've gone through a 20 year period where metabolism has been *passé*. I think that we are at a stage of knowledge now on cell death and mechanisms involved in cell death that we have some real possibilities for answers. I may be optimistic. I know that comment is self serving, but in terms of my own research, I've looked for years at the issue of chronic hypoxia and now realize that's irrelevant to the question of survival during short term anoxia. That was one of the points I tried to make. The anoxic model and the reoxygenation model are the ones that we have to deal with. We have real possibilities through basic research to come up with some more testable compounds. That may not provide us an immediate solution but it certainly is something that we have to invest in.

Dr. John Lemasters, University of North Carolina School of Medicine

I would like to address an issue related to our scientific tradition. As scientists, we are trained to look for the mechanisms underlying the particular problem at hand. Typically, we focus on one mechanism in an effort to establish how such a mechanism influences the outcome of some process, for example hypoxic injury. Similarly, drug companies are motivated in the same way. They are looking for that one drug that will cure a particular disease. Ideally, this drug acts by a single, well understood mechanism. In medical school we are taught the dangers of polypharmacy. Avoid using more than one drug so that adverse drug interactions, development of resistance to antibiotics, or simply overmedication does not develop. But for really sick patients, polypharmacy is the rule, not the exception. Patients with severe trauma are also very sick, and I think it is fundamentally wrong to expect that somehow there is going to be one drug, one mechanism, or one receptor to fill which will change profoundly the outcome of cellular injury. To rescue tissues from hypoxic and traumatic injury, we will need to go against the grain of our academic tradition. First, we have to find those things that work no matter how impractical they may seem at first. If you do not have something that works, practicality is no issue. Second, we will likely find that there are no silver bullets, no single mechanisms that explain everything. Many ideas have been expressed over the last two days that show promise to be important, even crucial in the development of irreversible cell injury. Sometimes our debate becomes the question of which idea is the best or most important. My belief is that we will find that all these ideas are important. Moreover in complicated clinical situations, we will need to apply all these ideas



to achieve a good outcome. In other words, each of the several mechanisms contributing to injury will have to be neutralized to see therapeutic success. Countering one mechanism alone may be completely ineffective, and we are prone to conclude wrongly that such a mechanism is unimportant in pathogenesis. Rather, multiple approaches and multiple drugs, i.e. polypharmacy, will be needed to treat very sick patients and their sick cells and tissues, because multiple measures are required to combat multiple pathogenetic mechanisms.

Dr. Constance Oliver

I'd like to ask a question to the panel and members of the audience. You've talked about tactical approaches or polypharmacy. Do we need different treatments for each organ?

Dr. Greg Fahy, Naval Medical Research Institute

What is the limiting organ in hypoxia and ischemia? Is it the brain? Should we be doing brain research?

Dr. Constance Oliver

Well, that probably depends on the type of injury. One thing I haven't talked about or alluded to is that the funding depends on requirements. The NIH funds brain research, so Department of Defense generally does not, except for a Congressionally-mandated plus-up of head trauma.

Dr. Sudhir V. Shah

There are a number of investigators around the country who are studying hypoxia or other areas of interest to the Navy in different organ systems such as the kidney, the brain, and the gut. Perhaps one of the roles the Armed Forces can play is to bring these investigators together and coordinate an effort centrally with the Navy. This is not the type of coordinated effort that is funded by NIH or other organizations and, therefore, may be a very special role for the Navy.

Dr. Constance Oliver

That's a point well taken. I'll say one of the purposes of this conference is to bring people together from different disciplines. It's not as broad and overarching as what you're suggesting but it's very valuable to bring people together with different perspectives in dealing with a common problem.

MAJ Verma

I agree with Dr. Shah's comments. I also wanted to add that we may learn a lot from nature's examples. There are animals that are well adapted to prolonged periods of hypoxia.

There are several groups doing work, Dr. Hallenbeck's group in particular, on animals that hibernate or estivate. We haven't heard a whole lot on the adaptive mechanisms the different organs employ in response to hypoxia, but we may learn a lot from studying animals which adapt. We heard about heat shock proteins and immediate early genes, but not much about how that relates to clinical settings. Perhaps there might be another focus on what's going on in the organs when soldier is down and out and hypoxic to adapt to that stress? Are we actually doing more harm by intervening in a certain way than we should? I also think that studies aimed toward the oxygen sensor and signal transduction mechanisms that detect changes in oxygen levels should be a main focus. Such a knowledge may allow us to modulate hypoxic responses therapeutically or prophylactically.

## Dr. Constance Oliver

There is another area that several speakers alluded to, the idea of preconditioning. Certainly in a military setting, where you know you're going to be sending people into a situation where they have a high potential of suffering from injury, is preconditioning practical? For instance, could induction of heat shock protein be useful for preconditioning? Is this something that we should be considering? Is this even practical?

## LCDR Rhee

My first question about preconditioning is will we give the guys some agent and heat them up before they go out and fight? In reality, the scientific question is about hypoxia and preconditioning for hypoxia. Is that a preconditioning or is that a priming? In certain circumstances that's more detrimental, but in other circumstances, if its preconditioning, it would probably be beneficial. So as a clinician, when I hear about that first initial phase, whether it be priming or preconditioning, In some instances the initial phase is protective and is called preconditioning, on the other hand the same initial phase is thought to be priming which is often detrimental. So in reality, which one is it? Priming or preconditioning? I wondered if some of the people involved with these types of projects could answer those types of questions for me?

## Dr. Constance Oliver

Would anybody care to comment? This is an area that we've obviously paid no real attention to and I think that's a good point. When talking about preconditioning, it sounds wonderful in a test tube or even in a rat to produce heat shock protein, but we could ultimately be making the situation worse in a trauma injury. I think that Ken's models show what you do to a septic animal is very different from one that's been traumatized.

## Dr. Kurt Henle, University of Arkansas

I just would like to point out that, of course, with a cellular stress response there are so many possible ways of inducing it non-traumatically that we really would not have to think

about preheating our soldiers. I think it would be much more realistic to think about taking some vitamins or retinoic acid, or something like this, which actually may help induce some organ-specific stress response. If you knew that you were going to be under attack, taking a pill, or something like this, would just statistically improve your chances for recovery and for survival and would be a cost effective and strategically logical approach to minimize the injury. But Dr. Lemasters is correct, in a sense, when he said we can't predict where the real breakthroughs will come. If we study the mechanisms and if we have the applications in mind, that's about as good as we can do. We really don't know where the benefits will accrue.

Dr. Constance Oliver

I appreciate your comment.

CDR Bennett

I think the notion of pretreatment is pretty exciting. It's far reaching and I challenge you not to get discouraged about approaching the concept of pretreatment. Pretreatment may be considered prophylactically, as we did with the weapons of mass destruction when we had significant intelligence on Iraqi forces and X amount of biological weapons fielded in their missiles. There was evidence that they had used them on Kurds during the Iran-Iraq war. FDA approved or not, we inoculated a significant portion of the ground forces for anthrax and botulinin. I have an interest in heat stress from a treatment standpoint, as well as a preventive measure. What did we do with Marines when they got there? No, we didn't stick them in warehouses to sit down and get ready for the war six or eight months later. They went right out into the 120 degrees dry inland heat. That's 120 degrees, very humid on the coast. They went right out and started working and got prepared from a heat acclimation standpoint. Heat acclimation is something that Marines understand. Heat acclimation actually increases the fluid volume requirements because one of the hallmarks of heat acclimation is not only reduction in core temperature and heart rate each day that you're exposed to that temperature, but also the total amount of sweat is actually increased. Fluid volume intake is actually increased in this kind of scenario. I wrote down a note when we had the heat shock protein presentation. I don't know the literature well in this particular area, but I'm questioning when the human gets heat acclimated, is there a protection from heat shock proteins associated with that as a precondition? If someone could answer that for me, I'd like to have a discussion with you. Also in weapons of mass destruction another condition or pretreatment is pyridostigmine bromide which is a pretreatment for particularly soman the nerve agent because of the aging concept. It is associated with the binding of soman to acetylcholinesterase and protects that particular enzyme to make it viable to cut down on the transmission of the acetylcholine on the postsynaptic cleft and all the associated cascade events occurred and acetylcholinesterases is essentially ineffective so there is another example. I caution you, the reason I'm talking about this is that we have a problem associated with Gulf War Syndrome. We're not authorized to use that term because there is nothing in the epidemiological data base that suggests that there ever was a syndrome. An animal model suggested that a possible problem with pretreatment or conditioning is pridostigmine bromide



as the pretreatment, Deet the agent you use on your skin to minimize insect infestation and malaria and so on, used in combination with permetherine which is another insect repellent spray for your clothing that soldiers use. The synergistic effect of all three of those potentially have a very high toxic response in the body, at least in the animal model and I think a lot of dollars are focussed on this to see if, in fact, there is any relevance to what might be the syndrome occurring in many of our soldiers and sailors.

Dr. Anne Murphy, George Washington University Medical Center

I think that the preconditioning idea is not necessarily only applicable in the combat field. I think that as a collaborator with clinicians there is certainly a constant concern. There are groups of people that we know are going into surgery when you know that certain parts of their body are going to be hypoxic for some period of time. I think the idea of preconditioning is not only applicable here, but for patients that are going to undergo certain types of surgeries. So we should keep in mind that it has more general applications.

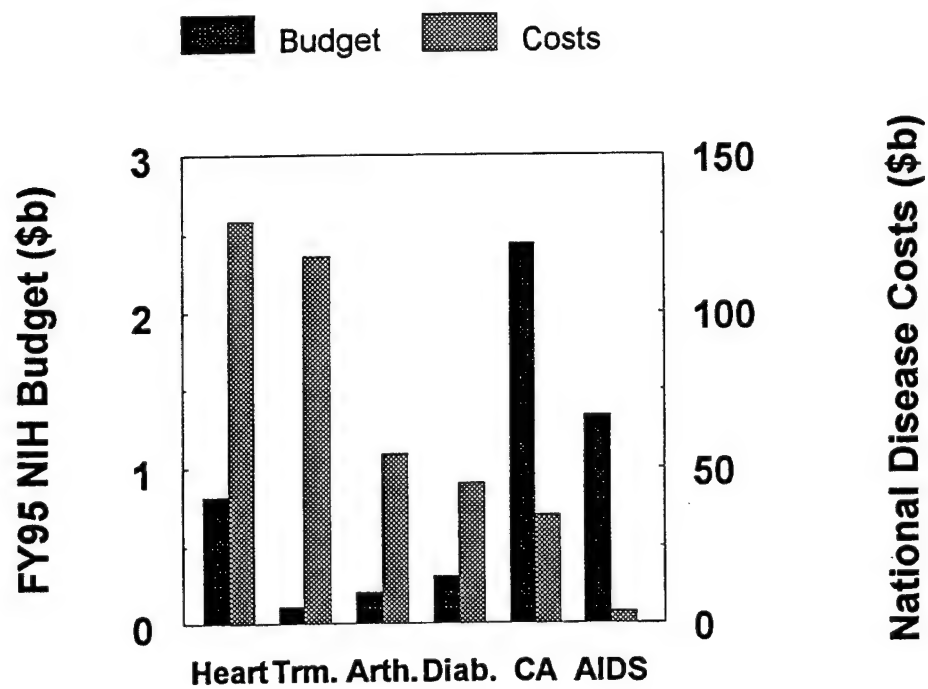
Dr. Constance Oliver

Thank you. Given the time, the program, and the schedule, I would very much like to thank both Anne and Thor for the excellent job they did in organizing the meeting. I know how much work it is. When a meeting runs smoothly it means that somebody's done a tremendous amount of work, so I'd like to thank them again for bringing us all together in what, for me, has been a very useful and very productive two days. I hope that all of you also found it useful. With that I'll turn it back to Thor. It's been a pleasure to meet some of you that I've only talked to on the telephone, and see some old friends again. Thank you.

Dr. Thor B. Nielsen

Thank you, Dr. Oliver. I'd like in particular to thank Dr. Murphy, without whose major efforts this symposium wouldn't have happened at all. I'd also like to thank Johanna Kidwell and Lisa Dalton for their tireless efforts. In a very real sense, I'd like to thank each and every one of you because a conference like this doesn't really work unless it affects the people who participate, unless those people make their own contribution, and it is reflected in their work. I hope that's been the case this time. Thank you very much. And perhaps we'll meet in another year.

## Research Investment and Disease Costs



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## SHORT COMMUNICATION

SALIVARY LEVELS OF  $\alpha_2$ -MACROGLOBULIN,  
 $\alpha_1$ -ANTITRYPSIN, C-REACTIVE PROTEIN, CATHEPSIN G  
AND ELASTASE IN HUMANS WITH OR WITHOUT  
DESTRUCTIVE PERIODONTAL DISEASEE. D. PEDERSON,<sup>1\*</sup> S. R. STANKE,<sup>2</sup> S. J. WHITENER,<sup>2</sup> P. T. SEBASTIANI,<sup>2</sup>  
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**Summary**—Five host-response indicators were measured by enzyme-linked immunosorbent assays on unstimulated whole saliva samples from 45 adults (19 male, 26 female). The participants were distributed among four dentate groups representing oral health (I), gingivitis (II), moderate periodontitis (III), and severe periodontitis (IV), and one group of edentulous volunteers (V). Levels of the host-response indicators varied widely, from zero, primarily with groups I and V, to relatively high values with groups II, III and IV. The levels ranged as follows:  $\alpha_2$ -macroglobulin, 0–4941 ng/ml;  $\alpha_1$ -antitrypsin, 2–2271 ng/ml; C-reactive protein, 0–472 pg/ml; cathepsin G, 0–6035 ng/ml; elastase, 0–164 ng/ml (free), 0–732 ng/ml (bound to  $\alpha_1$ -antitrypsin), and 0–318 ng/ml (bound to  $\alpha_2$ -macroglobulin). Statistical evaluation by planned contrasts showed that levels of host-response indicators for group I were significantly lower (except for  $\alpha_1$ -antitrypsin) than for groups II, III, and IV. A trend analysis of groups I–IV showed that mean scores (again, except for  $\alpha_1$ -antitrypsin) increased significantly in a positive, monotonic manner. Group V showed significantly lower values for elastase than in the other groups. The findings demonstrate that these factors can be detected in whole saliva and suggest that, except for  $\alpha_1$ -antitrypsin, their levels are directly related to an individual's periodontal status.

**Key words:** saliva, ELISA, C-reactive protein,  $\alpha_1$ -antitrypsin,  $\alpha_2$ -macroglobulin, polymorphonuclear leucocytes, cathepsin G, elastase.

Human whole saliva contains a wide variety of proteins, including enzymes (reviewed by Ellison, 1979) derived from micro-organisms, salivary glands, gingival crevicular fluid, dietary constituents and polymorphonuclear neutrophils (Chauncey, 1961; Makinen, 1966a, b; Lindqvist *et al.*, 1974). Nakamura and Slots (1983) identified 76 enzyme activities in whole saliva by means of the API ZYM semiquantitative system. These activities, many of which originated from micro-organisms, were found to be higher in periodontally diseased than in healthy individuals. Mandel (1991) has reviewed salivary markers of periodontal disease susceptibility and activity, but no studies of the host-response indicators of the present report were cited.

Gingival crevicular fluid contains various host-response indicators, such as: (1) from neutrophil

granules, the enzymes cathepsin G (Suomalainen, 1992) and elastase (Ohlsson *et al.*, 1974), (2) the elastase inhibitors,  $\alpha_2$ -macroglobulin and  $\alpha_1$ -antitrypsin (Ohlsson *et al.*, 1974; Asman *et al.*, 1981), and (3) the complement-activating factor, C-reactive protein (Sibraa *et al.*, 1991).

Many recent studies have explored the association of crevicular-fluid factors with gingivitis and with more advanced stages of periodontal disease (reviewed by Curtis, 1991; Lamster, 1991). Positive relations have been reported for neutrophil elastase (Ohlsson *et al.*, 1974; Kowashi *et al.*, 1979; Giannopoulou *et al.*, 1992; Gustafsson *et al.*, 1992; Palkanis *et al.*, 1992).

Several studies of  $\alpha_2$ -macroglobulin and  $\alpha_1$ -antitrypsin (Ohlsson *et al.*, 1974; Condacci *et al.*, 1982; Giannopoulou *et al.*, 1992; Adonogianaki *et al.*, 1994) have shown increased levels in crevicular fluid associated with gingivitis. With more advanced stages of periodontal disease, the concentration of  $\alpha_2$ -macroglobulin was found to be lower in the fluid from sites with tissue destruction than from healthy

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**Abbreviations:** CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; GI, gingival index; PBS, phosphate-buffered saline; PD, probing depth.

sites (Skaleric *et al.*, 1986; Sibraa *et al.*, 1991; Gustafsson, Asman and Bergström, 1994). On the other hand, the concentration of  $\alpha_1$ -antitrypsin was higher in adult periodontitis or rapidly progressive periodontitis than in gingivitis (Huynh *et al.*, 1992). Sibraa *et al.* (1991) also reported that levels of C-reactive protein in gingival crevicular fluid from patients with periodontitis did not vary significantly in healthy or diseased sites.

In this preliminary study, we have measured levels of these indicators in whole saliva from adults in order to determine whether such levels can be related to periodontal disease status.

Forty-five (19 male, 26 female) participants were selected from patients visiting the Northwestern University Dental School Clinic. They ranged in age from 23 to 80 yrs and in periodontal status from healthy to chronically diseased or edentulous. None had had dental treatment or antibiotic therapy within the 6 months preceding the current clinic visit. The patients presented, as scheduling permitted, between the hours of 9–12 a.m. and 2–5 p.m. Individuals who had serious systemic diseases (such as diabetes, cancer or AIDS) were excluded from the study.

Information was obtained for each participant, except for the edentulous, on American Dental Association periodontitis case type, Gingival Index (Silness and Løe, 1964), and probing depths at six sites around each tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual).

A 1–2 ml sample of unstimulated whole saliva (drool) was collected from each participant after a preliminary oral rinse with water. Each sample was diluted with an equal volume of phosphate-buffered saline, pH 7.0 (Voller *et al.*, 1976) containing 0.08% sodium azide and 0.01 M EDTA, and the sample was chilled with ice and refrigerated at 4°C until the assays could be done, within 24–48 h. No discoloured samples were used.

Measurements were made on each salivary sample by ELISA for levels of polymorphonuclear neutrophil cathepsin G,  $\alpha_2$ -macroglobulin,  $\alpha_1$ -antitrypsin, C-reactive protein and elastase. The levels of elastase were determined for the free enzyme, as well as for the enzyme bound to  $\alpha_2$ -macroglobulin and  $\alpha_1$ -antitrypsin. (It must be recognized that this application of the ELISA simply measured amounts rather than activities of the enzymes cathepsin G and elastase.)

The ELISA procedures were generally similar for each of the factors under study, and were done with Xenobind (Xenopore Corp., Saddle Brook, NJ) 96-well microtitre plates, which are designed to bind an initially introduced protein on to their surfaces. The initial protein was a capture antibody in every case except cathepsin G, for which the first step was the addition of the samples and standards directly to the wells. Primary and secondary antibodies were then added in sequence, as employed in the double-antibody sandwich procedure of Singh and Tingle (1982). For measurements of the complexes, elastase/ $\alpha_2$ -macroglobulin and elastase/ $\alpha_1$ -antitrypsin, capture antibodies for the macroglobulin and antitrypsin were employed initially, followed by the addition of anti-human neutrophil elastase as the primary detection antibody. The basic steps in the procedure have

been described by Geivelis *et al.* (1993), and the reagents employed for the specific measurements are listed in Table 1.

The participants in this study, except for the six edentulous individuals, were categorized according to periodontal status into four groups designated (I) healthy, (II) gingivitis, (III) mild-to-moderate periodontitis, (IV) moderate-to-severe periodontitis. The mean gingival index (GI) and mean probing depth (PD) for each individual were used to determine the group mean. These values, which show the expected positive trends of increase in relation to disease severity, were as follows: group I—GI = 0.67, PD = 2.32; group II—GI = 0.77, PD = 2.56; group III—GI = 1.55, PD = 3.49; group IV—GI = 1.90, PD = 4.17. The edentulous individuals were included as a control group that was free of periodontal disease and able to provide saliva without the contribution of gingival crevicular fluid.

Table 2 presents the findings on mean levels of the host-response factors under study that could be detected in the saliva samples from each group. It is evident that group I (healthy) and group V (edentulous) generally showed much lower mean levels of the test components than were found for groups II, III and IV. Individual values ranged very widely, from zero (below the level of detectability) primarily with group I individuals to relatively high values within groups II, III, and IV. The most marked contrast was between the healthy (group I) and the group II individuals with gingivitis, the gingivitis group showing factor levels much closer to, or even overlapping, those of corresponding categories in groups III and IV.

The data exhibited marked skew so, for purposes of inferential statistical methods, raw data were subjected to a log transformation (where 1 was added to each data-point before transformation). A planned contrast was performed on the healthy group (I) versus the diseased groups (II, III and IV). A trend analysis was also made to test for linear or quadratic effects across the dentate groups (I–IV). Another planned contrast was performed on the edentulous group (V) versus the dentate groups (I, II, III and IV).

Table 3 presents the results of these statistical tests. The first set, the planned contrast, showed that the differences between the healthy group (I) and the groups with periodontal diseases (II, III, IV) were significant for all of the factors tested except  $\alpha_1$ -antitrypsin. The linear and quadratic trend analyses (Table 3; a, b, c and d) showed similar patterns of significance for (a) and (d), which demonstrated a general increase in salivary levels for all of the factors with increasing severity of periodontal disease, except again for  $\alpha_1$ -antitrypsin. However, less significant differences were found among the factors for the tests (b) and (c). Apart from the significantly lower levels of neutrophil elastase (probably due to relatively low numbers of oral polymorphonuclear neutrophils), the edentulous showed no significant differences from the remaining participants in salivary levels of the host-response factors (third test).

As noted earlier, the data for mean levels of  $\alpha_1$ -antitrypsin (Table 2) showed an increasing trend across groups I–IV, although the differences among the groups were not significant. Our findings are



Table 1. Reagents employed for ELISA

Host-factor	Standards	Antibody (capture and detection)		
		Capture	Primary detection	Secondary detection
1. PMN-CG	2-1000 ng/ml PMN-CG** in PBS + 0.04% sodium azide	—	Rabbit anti-human* PMN-CG diluted 1:5000 with PBST + 1% BSA	Goat anti-rabbit** IgG/alkaline phosphatase diluted 1:2500 with PBST + 1% BSA
2. A2M	2-1000 ng/ml A2M* in PBSI + 1% BSA	Rabbit anti-human** A-2M diluted 1:5000 with PBS	Sheep anti-human** A-2M diluted 1:2000 with PBST + 1% BSA	Donkey anti-sheep** IgG/alkaline phosphatase diluted 1:2500 with PBST + 1% BSA
3. AAT	0.05-1000 ng/ml AAT* in PBST + 1% BSA	Rabbit anti-human AAT* diluted 1:2000 with PBS	Sheep anti-human*** AAT diluted 1:2000 with PBST + 1% BSA	Donkey anti-sheep** IgG/alkaline phosphatase diluted 1:2000 with PBST + 1% BSA
4. CRP	0.05-25 pg/ml CRP†† in PBST + 1% BSA	Sheep anti-human† CRP diluted 1:2000 with PBS	Rabbit anti-human††† CRP diluted 1:2000 with PBST + 1% BSA	Goat anti-rabbit** IgG/alkaline phosphatase diluted 1:2000 with PBST + 1% BSA
5. PMN-E	3-400 ng/ml PMN-E* in PBST + 1% BSA	Rabbit anti-human* PMN-E diluted 1:3000 with PBS	Goat anti-human* PMN-E diluted 1:5000 with PBST + 1% BSA	Rabbit anti-goat** IgG/alkaline phosphatase diluted 1:2000 with PBST + 1% BSA

\*Calbiochem Corp., La Jolla, CA.

\*\*Sigma Chemical Co., St Louis, MO.

\*\*\*The Binding Site, University of Birmingham Research Institute, U.K.

†Cooper Biomedical, Westchester, PA.

††Behring Diagnostics Corp., La Jolla, CA.

†††DAKO, Denmark.

PMN-CG, polymorphonuclear neutrophil cathepsin G; A2M,  $\alpha_2$ -macroglobulin; AAT,  $\alpha_1$ -antitrypsin; BSA, bovine serum albumin; CRP, C-reactive protein; PBST, phosphate-buffered saline/0.05% Tween-20; PMN-E, polymorphonuclear neutrophil elastase.

Table 2. Levels of salivary immune-response indicators among healthy, periodontally diseased and edentulous individuals

Group	PMN-CG (ng/ml)	A2M (ng/ml)	AAT (ng/ml)	CRP (pg/ml)	PMN-E (ng/ml)	PMN-E	
						AAT (ng/ml)	A2M (ng/ml)
<i>I. Healthy (n = 5)</i>							
Mean	24.1	38.1	60.1	6.7	22.3	23.7	12.0
SD	20.8	32.3	100.5	6.5	32.4	15.5	15.7
Range	0-57	0-89	5-240	0-17	0-75	0-37	0-39
<i>II. Gingivitis (n = 8)</i>							
Mean	409.9	398.1	212.8	75.1	40.9	150.0	28.8
SD	712.4	482.9	291.2	163.1	23.5	240.7	25.8
Range	0-2101	24-1186	12-804	1-472	9-66	0-732	3-78
<i>III. Mild-to-moderate periodontitis (n = 18)</i>							
Mean	688.5	611.6	291.2	43.4	69.6	130.4	66.0
SD	1436.6	919.9	415.7	49.6	49.8	165.2	82.9
Range	24-6035	5-2919	5-1277	2-190	17-164	15-717	6-318
<i>IV. Moderate-to-severe periodontitis (n = 8)</i>							
Mean	541.8	873.0	380.2	80.4	51.7	77.6	59.7
SD	1009.7	1664.6	775.0	104.3	45.0	55.7	76.3
Range	42-3029	59-4941	2-2271	0-306	14-152	23-201	6-238
<i>V. Edentulous (n = 6)</i>							
Mean	44.8	132.4	68.7	10.2	17.4	0.5	1.6
SD	37.2	92.6	104.0	5.3	16.9	1.2	3.2
Range	18-115	16-216	4-278	3-18	0-38	0-3	0-8

Abbreviations as in Table 1.

similar to those of Adonogianaki *et al.* (1992), who measured levels of  $\alpha_2$ -macroglobulin and  $\alpha_1$ -antitrypsin in crevicular fluid from healthy, gingivitis, and periodontitis sites of humans. Although they found that levels of  $\alpha_2$ -macroglobulin differed significantly among the three types of sites, the levels of  $\alpha_1$ -antitrypsin were not significantly different. They point out that, while  $\alpha_2$ -macroglobulin is considered to be both serum derived and locally produced by fibroblasts and macrophages,  $\alpha_1$ -antitrypsin is predominantly serum derived.

The most pronounced increase in levels of the factors was observed between periodontally healthy individuals (group I) and those with gingivitis (group II). The factor levels for groups II, III and IV differed less, and even overlapped in some cases, among corresponding categories. This general pattern agrees well with the patterns for mean levels in crevicular fluid of neutrophil elastase (Gustafsson *et al.*, 1992; Giannopoulou *et al.*, 1994), and of  $\alpha_2$ -macroglobulin

and  $\alpha_1$ -antitrypsin (Adonogianaki *et al.*, 1992), for similar groups of individuals. In each case a marked increase in mean factor level was found when groups of healthy and 'with gingivitis' individuals were compared. These observations suggest that the body's defence mechanisms are readily responsive even to challenges of incipient periodontal disease, and that such effects can be detected in saliva as well as in gingival crevicular fluid.

Some studies (Wewers, Herzyk and Gadek, 1988; Giannopoulou *et al.*, 1992) have reported that neutrophil elastase cannot be detected by immuno-reactive techniques when complexed with  $\alpha_2$ -macroglobulin, in contrast to the present findings. An explanation may be that, in each of the foregoing studies, the primary-detection antihuman neutrophil elastase antibodies had been obtained from rabbits, while we used goat anti-human neutrophil elastase antibodies, which may interact with epitopes on the elastase molecule that fail to react with the

Table 3. Statistical evaluation of group comparisons (*p* values)

Group contrasts	PMN-CG	A2M	AAT	CRP	PMN-E	PMN-E	
						AAT	A2M
I vs II, III, IV*	0.0035	0.0032	n.s.	0.0427	0.0009	0.0164	0.0087
I, II, III, IV**							
(a) Linear [LIN]	0.0024	0.0045	n.s.	0.0238	0.0033	0.0281	0.0063
(b) Quadratic [QUAD]	n.s.	n.s.	n.s.	n.s.	0.0140	n.s.	n.s.
(c) QUAD condition on LIN [QCL]	n.s.	n.s.	n.s.	n.s.	0.0350	n.s.	n.s.
(d) LIN + QCL	0.0001	0.0002	n.s.	0.0064	0.0000	0.0023	0.0005
V vs I, II, III, IV***	n.s.	n.s.	n.s.	n.s.	0.0355	0.0000	0.0000

\*Teeth present—health vs disease.

\*\*Teeth present—linear and quadratic trends.

\*\*\*Teeth absent vs teeth present.

n.s., Not significant. Other abbreviations as in Tables 1 and 2.

rabbit antibodies, while epitopes that can react with the rabbit antibodies are not accessible in the complex.

The wide range observed in the whole-saliva levels of the host-response indicators could result in part from differences in disease activity and crevicular fluid flow at the gingival sites, as well as from variations in the number of polymorphonuclear neutrophils migrating into the mouth. Transient factors, such as oral ulcers or injuries, could also greatly affect saliva composition.

Although individuals with obvious disorders were not included in our study, it must be recognized that subtle systemic disorders among our test participants could also have affected their salivary levels of the host-response indicators under study (Mandel, 1990).

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Bactericidal Effects of PMN Azurophil Granule Components on *Treponema denticola*.  
E. D. PEDERSON\*, D. W. TURNER, C. T. GLYNN, C. MERRITT and P. L. TECK  
[The Naval Dental Research Institute, Building 1-H, Great Lakes, IL 60088-5259, USA].

Current evidence indicates that polymorphonuclear neutrophils [PMN] and other phagocytes play a major role in controlling microorganisms in the periodontium [Miyasaki, 1991], thereby protecting the host against periodontal pathogens. Levels of the pathogenic oral spirochete, *Treponema denticola* [Td], have been shown to increase as periodontal pocket depth increases [Simonson, 1988]. The aim of this study was to determine whether certain PMN factors were bactericidal for Td. Purified PMN factors were tested on Td strains ATCC 35405, 33521 and 33520 [serovars a, b and c, respectively] grown in Spirolate & 1186 broths (1:1, v/v). Elastase [PMNE], lactoferrin [LACTO], lysozyme [LYSO], defensins 1 & 2 [HNP-1&2], cathepsin-G [C-G], heat-inactivated C-G [C-HI] and the microbicidal peptide from C-G [C-M] at 25, 50 and 100 µg/ml concentrations were combined with  $1 \times 10^5$  Td cells. C-G was also used at 1.63, 3.13, 6.25 and 12.5 µg/ml concentrations. Td and PMN factors were placed into 10 mM sodium phosphate buffer, pH 7.5. Ten µl of the Td, 10 µl buffer and 20 µl of each PMN factor were combined in sterile microcentrifuge tubes. Following a 1-hr incubation at 37°C, 1/40 of the mixture was transferred to culture flasks containing 25 ml of the 1:1 broth and 0.75% Sea Plaque agar [Chan, 1993 & Pederson, 1994]. After incubation at 37°C the numbers of Td colonies/flask were determined for control and treated Td from three replicate flasks. Kills were expressed as percent of control growth. C-G ( $p < 0.001$ ) and C-HI ( $p < 0.001$ ) were 100% microbicidal at the 25, 50 and 100 µg/ml concentrations, and C-G was microbicidal at even lower levels. A concurrent study found the levels of C-G in gingival crevicular fluid to correlate with the µg Td in plaque ( $r = .2837$ ,  $df = 298$ ,  $p < 0.0001$ ). LYSO ( $p < 0.005$ ), C-M ( $p < 0.05$ ), and PMNE, HNP-1 and HNP-2 ( $p < 0.001$ -NS) demonstrated moderate to limited (selective) killing. LACTO was not microbicidal for Td at the 25, 50 or 100 µg/ml levels. Cathepsin-G and heat-inactivated cathepsin-G demonstrated microbicidal activity against all three Td serovars, when compared to controls. Supported by NMRDC, Projects 061152N MR00001.001-0064 and 061153N MR04120.002-0051.

Bactericidal Activity of PMN Factors on *Treponema denticola*. E. D. PEDERSON\*,  
D. W. TURNER, S. Z. SCHADE, and L. G. SIMONSON [Naval Dental Research  
Institute, Building 1-H, Great Lakes, Illinois 60088-5259, USA].

Current evidence indicates that polymorphonuclear neutrophils [PMNs] and other phagocytes play a major role in controlling microorganisms in the periodontium [Miyasaki, 1991], thereby protecting the host against periodontal pathogens and diseases. Levels of the pathogenic oral spirochete *Treponema denticola* [Td] have been shown to increase as periodontal pocket depth increases [Simonson, 1988]. The aim of this study was to determine whether bactericidal actions of certain PMN factors and Td could be detected *in vitro*. Four purified PMN factors, elastase [PMNE], myeloperoxidase [MPO], lysozyme [LYSO] and cathepsin-G [Cath-G] were tested on Td strains ATCC 35405 and VPI D39DP1. Bacterial cell concentrations, determined spectrophotometrically at 660 nm, were  $1-5 \times 10^7$  Td cells/ml. Td and PMN factors were placed in saline containing 20 mM MgCl<sub>2</sub> for testing. Treatments consisted of combining 50  $\mu$ l of each Td suspension [ $0.5-2.5 \times 10^6$  cells], and 0.23, 1.39 or 8.33  $\mu$ g of each factor [50  $\mu$ l] in duplicate wells of low background fluorescence 96-well microtiter plates. Live/Dead<sup>R</sup> BacLight Viability dyes and a Fluoroskan II microplate reader were employed to distinguish live from dead [L vs D] Td. L/D ratios of three replicate readings made at 30 min intervals, compared to controls, were used to determine Td killing. Of the four PMN factors tested, only Cath-G demonstrated detectable microbicidal activity [ $p < 0.01$  to  $p < 0.001$ ] for the two Td isolates when compared to PMNE, MPO and LYSO, at the 0.23, 1.39 and 8.33  $\mu$ g levels. The results indicate that Td is more sensitive to killing by cathepsin-G than by the other factors examined in this pilot study. Results were verified by the DAPI/PI method of Turner, 1995. Supported by the Office of Naval Research, Naval Medical Research and Development Command, Bethesda, Maryland, project number 0601152N MR00001.001-0064.



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

REGION 5  
77 WEST JACKSON BOULEVARD  
CHICAGO, IL 60604-3590

REPLY TO THE ATTENTION OF:

WT-16J

17 May 1996

Participant in Water Pollution Performance  
Evaluation Study Thirty-Five (WP035),  
through Region Five.

Reference: Evaluation of Your Laboratory's Performance  
in WP035.

Dear Participant:

The results your laboratory reported from its analysis of samples distributed for this WP035 study have been evaluated. This evaluation is presented on computer-printout sheets, which are enclosed. Also enclosed is a copy of Paul W. Britton's memorandum of 30 April, 1996 concerning this study, and an explanation of the evaluation terms used.

3,000 laboratories, nationwide, participated in this study; a summary report of the results from these laboratories is enclosed. Additional summary reports for state laboratory and for federal laboratory participants are enclosed; these cover those respective laboratory groups.

Yours truly,

*Robert J. Gnaedinger Jr.*

Robert J. Gnaedinger, Jr., Ph.D., Chemist  
Standards & Applied Sciences Branch, Water Division  
Telephone: 312/353-2975

encl

cc: Joan Karnauskas  
Chief, SASB

RJGJR/rg



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY  
NATIONAL EXPOSURE RESEARCH LABORATORY  
CINCINNATI, OH 45268

Date: April 30, 1996

OFFICE OF  
RESEARCH AND DEVELOPMENT

SUBJECT: Results from Water Pollution Performance  
Evaluation Study 35 (WP035)

FROM: Paul W. Britton, Statistician *Paul W. Britton*  
National Water Quality Assurance Programs Branch  
Ecological Exposure Research Division

TO: Quality Assurance Coordinators/Officers  
Project Officers  
Interested Laboratories

THRU: Robert L. Graves, Chief *RJG*  
National Water Quality Assurance Programs Branch  
Ecological Exposure Research Division

WP035 has been completed by NERL-Cincinnati in its continuing evaluation of the performance of USEPA, state and other selected laboratories for 80 water pollution analytes.

Addressees that nominated a group of laboratories directly to NERL-Cincinnati will find:

- 1) a single copy of the performance evaluation report for each laboratory they nominated and related study information;
- 2) a 3.5" diskette containing the study results as personal computer (PC) files that have been compressed using PKZIP, and directions regarding use of the diskette;
- 3) a printed list of the participating laboratories within their jurisdiction, including their assigned codes and identification of which specific addressee must distribute the report, etc., to EACH laboratory.

The diskette constitutes their file copy of the study results and the printed copy is for distribution to each of their participating laboratories (also see paragraph at the top of page 2 regarding which coordinator has the primary responsibility for distributing the report to each specific lab). As a study coordinator, it is also their responsibility to provide study results to any interested state or regional official within their jurisdiction that needs the information and is not listed as an addressee above. Study coordinators may distribute copies of any reports, files or other study information, as they consider necessary.

US ENVIRONMENTAL PROTECTION AGENCY  
ENVIRONMENTAL MONITORING SYSTEMS LABORATORY-CINCINNATI

TERMS USED FOR WATER POLLUTION (WP) LABORATORY PERFORMANCE EVALUATION STUDIES

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TRUE VALUES: A THEORETICALLY CALCULATED VALUE BASED UPON CAREFUL WEIGHT AND VOLUME MEASUREMENT OF CONSTITUENTS. PLEASE NOTE THAT, FOR SOME ANALYTES, THIS VALUE IS QUITE DIFFERENT FROM THE RESULT THAT CAN BE EXPECTED WHEN THE STUDY SAMPLE IS ANALYZED. WHEN UNAVOIDABLE, THIS MAY BE A REFERENCE VALUE THAT REPRESENTS ACHIEVABLE RESULTS.

ACCEPTANCE LIMITS: A 99% PREDICTION INTERVAL CALCULATED FROM AVAILABLE PERFORMANCE EVALUATION DATA OF EPA AND STATE LABORATORIES.

BY DEFINITION THE ANALYTIC RESULTS FROM A LABORATORY PRODUCING VALID DATA SHOULD FALL WITHIN ACCEPTANCE LIMITS 99 TIMES OUT OF 100.

WARNING LIMITS: A 95% PREDICTION INTERVAL PRODUCED IN THE SAME WAY AS THE ACCEPTANCE LIMITS.

DATA FALLING OUTSIDE THESE LIMITS BUT INSIDE THE ACCEPTANCE LIMITS SHOULD BE REVIEWED FOR POSSIBLE PROBLEMS, BUT SUCH DATA SHOULD NOT NECESSARILY BE CONSIDERED UNACCEPTABLE.

---

MEANING OF THE PERFORMANCE EVALUATION COMMENTS:

ACCEPT.: THE REPORTED VALUE WAS WITHIN ALL LIMITS.

CK. FOR ERR.: THE REPORTED VALUE WAS WITHIN THE ACCEPTANCE LIMITS, I.E., TECHNICALLY ACCEPTABLE, BUT SINCE THE VALUE WAS OUTSIDE OF THE WARNING LIMITS A MARGINAL PROBLEM MAY EXIST.

NOT ACCEPT.: THE REPORTED VALUE WAS BEYOND THE ACCEPTANCE LIMITS.

UNUSABLE: THE VALUE WAS REPORTED AS A "LESS THAN" OR "GREATER THAN" VALUE AND COULD NOT BE QUANTITATIVELY JUDGED.

---

ANY PERFORMANCE EVALUATION OTHER THAN ACCEPTABLE REQUIRES A REVIEW BY THE LABORATORY TO IDENTIFY POSSIBLE DEFICIENCIES OR ERRORS THAT NEED CORRECTING.



Summary Report for All Participants  
USEPA Water Pollution Study WP035

Report: PE-010  
Page: 1  
Date: 16APR96

Requesting Office: ALL

Total Participants: 3,000

Sample Number	True Value	Results Reported	Usable	Accept.	Check For Error	Not Accept.
TRACE METALS IN MICROGRAMS/LITER						
001-ALUMINUM						
01	321	1,029	1,024	865	43	116
02	1500	1,030	1,030	881	55	94
002-ARSENIC						
01	193	1,115	1,115	895	77	143
02	571	1,116	1,116	925	69	122
003-BERYLLIUM						
01	190	996	996	887	43	66
02	541	995	995	852	64	79
004-CADMIUM						
01	52.6	1,242	1,238	1,084	74	80
02	401	1,242	1,242	1,127	40	75
005-COBALT						
01	28.1	878	862	714	52	96
02	624	885	885	760	52	73
006-CHROMIUM						
01	17.0	1,266	1,231	973	83	175
02	880	1,277	1,276	1,108	56	112
007-COPPER						
01	86.7	1,262	1,258	1,050	66	142
02	370	1,263	1,263	1,107	65	91
008-IRON						
01	30.4	1,166	1,117	850	80	187
02	464	1,180	1,179	850	114	215
009-MERCURY						
01	3.10	1,031	1,029	865	46	118
02	11.6	1,028	1,028	807	59	162
010-MANGANESE						
01	401	1,137	1,137	984	59	94
02	881	1,138	1,138	914	100	124
011-NICKEL						
01	496	1,230	1,229	1,043	89	97
02	611	1,231	1,230	1,077	62	91
012-LEAD						
01	297	1,267	1,267	1,050	85	132
02	399	1,266	1,265	1,040	75	150
013-SELENIUM						
01	522	1,083	1,083	954	47	82
02	978	1,084	1,084	973	40	71
014-VANADIUM						
01	211	834	832	690	54	88
02	811	837	837	679	69	89
015-ZINC						
01	71.9	1,227	1,225	1,001	55	169
02	1800	1,230	1,229	1,079	45	105

Summary Report for All Participants  
USEPA Water Pollution Study WP035

Report: PE-01  
Page:  
Date: 16APR9

Requesting Office: ALL

Total Participants: 3,00

Sample Number	True Value	Results Reported	Usable	Accept.	Check For Error	No Accept
016-ANTIMONY						
03	370	954	954	874	28	52
04	570	956	956	873	36	47
017-SILVER						
03	180	1,176	1,176	1,045	35	96
04	340	1,176	1,176	1,012	59	105
018-THALLIUM						
03	83.3	921	913	764	51	98
04	365	920	920	780	57	83
074-MOLYBDENUM						
03	130	893	889	728	62	99
04	310	895	893	768	42	83
075-STRONTIUM						
03	3.55	532	486	378	25	83
04	96.0	543	541	475	21	45
076-TITANIUM						
03	115	593	592	460	57	75
04	270	593	592	485	27	80
MINERALS IN MILLIGRAMS/LITER (EXCEPT AS NOTED)						
019-PH-UNITS						
03	4.30	2,391	2,390	1,973	130	287
04	5.50	2,392	2,391	1,974	100	317
020-SPEC. COND. (UMHOS/CM AT 25 C)						
01	916	1,334	1,334	1,105	63	166
02	586	1,335	1,335	1,078	61	196
021-TDS AT 180 C						
01	553	1,256	1,256	1,057	73	126
02	311	1,252	1,252	1,161	33	58
022-TOTAL HARDNESS (AS CaCO3)						
01	330	1,169	1,169	971	66	132
02	101	1,170	1,170	956	68	146
023-CALCIUM						
01	104	1,118	1,118	962	51	105
02	6.39	1,112	1,112	866	63	183
024-MAGNESIUM						
01	17.0	1,062	1,062	875	64	123
02	20.6	1,058	1,058	891	73	94
025-SODIUM						
01	14.2	1,044	1,044	807	81	156
02	54.3	1,042	1,042	779	93	170
026-POTASSIUM						
01	21.0	1,002	1,002	795	79	128
02	38.3	1,003	1,003	805	51	147
027-TOTAL ALKALINITY (AS CaCO3)						
01	20.0	1,237	1,236	1,011	52	173
02	72.0	1,240	1,239	1,038	65	136

Summary Report for All Participants  
USEPA Water Pollution Study WP035

Report: PE-010  
Page: 3  
Date: 16APR96

Requesting Office: ALL

Total Participants: 3,000

Sample Number	True Value	Results Reported	Usable	Accept.	Check For Error	Not Accept.
028-CHLORIDE						
01	241	1,272	1,271	988	87	196
02	72.7	1,272	1,271	1,030	91	150
029-FLUORIDE						
01	3.50	997	996	711	90	195
02	1.35	997	996	755	87	154
030-SULFATE						
01	18.0	1,134	1,133	979	50	104
02	86.4	1,132	1,132	938	79	115
NUTRIENTS IN MILLIGRAMS/LITER						
031-AMMONIA-NITROGEN						
01	19.0	1,335	1,335	1,133	61	141
02	1.40	1,333	1,333	1,130	65	138
032-NITRATE-NITROGEN						
01	8.31	1,211	1,211	1,036	48	127
02	0.390	1,207	1,195	946	49	200
033-ORTHOPHOSPHATE						
01	.0560	1,073	1,026	792	27	207
02	2.80	1,078	1,078	867	49	162
034-KJELDAHL-NITROGEN						
03	0.540	901	881	712	51	118
04	7.80	903	903	746	41	116
035-TOTAL PHOSPHORUS						
03	0.574	1,091	1,090	876	43	171
04	6.08	1,091	1,091	876	32	183
DEMANDS IN MILLIGRAMS/LITER						
036-COD						
01	236	1,059	1,058	865	67	126
02	101	1,060	1,060	939	31	90
037-TOC						
01	93.1	567	567	486	27	54
02	40.1	568	568	509	16	43
038-5-DAY BOD						
01	141	1,421	1,419	1,329	43	47
02	62.5	1,411	1,410	1,297	48	65
102-CARBONACEOUS BOD						
01	117	716	716	692	18	6
02	51.6	722	722	673	21	28
PCB'S IN MICROGRAMS/LITER						
040-PCB-AROCOR 1016/1242						
01	Not Pres	173	173	0	0	173
02	Not Pres	41	41	0	0	41
042-PCB-AROCOR 1232						
01	2.76	597	583	511	27	45
02	Not Pres	16	16	0	0	16

Summary Report for All Participants  
USEPA Water Pollution Study WP035

Report: PE-01C  
Page: 4  
Date: 16APR96

Requesting Office: ALL

Total Participants: 3,000

Sample Number	True Value	Results Reported	Usable	Accept.	Check For Error	Not Accept.
044-PCB-AROCOLOR 1248						
01	Not Pres	12	12	0	0	12
02	4.26	702	701	638	19	44
045-PCB-AROCOLOR 1254						
01	Not Pres	6	6	0	0	6
02	Not Pres	10	10	0	0	10
046-PCB-AROCOLOR 1260						
01	Not Pres	1	1	0	0	1
02	Not Pres	1	1	0	0	1
PCB'S IN OIL IN MILLIGRAMS/KILOGRAM						
099-PCB IN OIL- 1016/1242						
01	42.3	690	690	625	23	42
02	Not Pres	8	8	0	0	8
100-PCB IN OIL- 1254						
01	Not Pres	5	5	0	0	5
02	Not Pres	6	6	0	0	6
101-PCB IN OIL- 1260						
01	Not Pres	11	11	0	0	11
02	12.7	691	690	606	30	54
PESTICIDES IN MICROGRAMS/LITER						
047-ALDRIN						
01	3.11	712	712	673	19	20
02	0.243	711	711	634	37	40
048-DIELDRIN						
01	4.51	711	711	598	36	77
02	1.62	712	712	622	41	49
049-DDD						
01	5.67	718	718	614	35	69
02	1.94	717	717	581	48	88
050-DDE						
01	3.76	716	716	596	41	79
02	1.42	715	715	604	38	73
051-DDT						
01	6.46	718	718	589	47	82
02	1.76	719	719	604	41	74
052-HEPTACHLOR						
01	2.85	714	714	664	27	23
02	0.278	712	710	638	29	43
053-CHLORDANE						
03	12.3	694	694	612	25	57
04	1.36	691	690	521	46	123
078-HEPTACHLOR EPOXIDE						
01	2.20	715	715	609	45	61
02	0.284	714	712	606	42	64

Summary Report for All Participants  
USEPA Water Pollution Study WP035

Report: PE-010  
Page: 5  
Date: 16APR96

Requesting Office: ALL

Total Participants: 3,000

Sample Number	True Value	Results Reported	Usable	Accept.	Check For Error	Not Accept.
VOLATILE HALOCARBONS IN MICROGRAMS/LITER						
054-1,2 DICHLOROETHANE						
01	56.3	911	910	856	16	38
02	12.2	910	909	847	27	35
055-CHLOROFORM						
01	64.8	920	920	815	50	55
02	14.2	919	919	811	47	61
056-1,1,1 TRICHLOROETHANE						
01	63.7	920	920	855	24	41
02	16.2	918	918	857	30	31
057-TRICHLOROETHENE						
01	72.3	919	919	852	24	43
02	16.1	919	919	846	36	37
058-CARBONTETRACHLORIDE						
01	29.0	918	918	854	24	40
02	9.36	916	916	840	33	43
059-TETRACHLOROETHENE						
01	73.6	922	922	843	33	46
02	10.4	924	924	857	28	39
060-BROMODICHLOROMETHANE						
01	55.6	912	912	843	34	35
02	14.6	913	913	821	41	51
061-DIBROMOCHLOROMETHANE						
01	48.5	914	914	827	34	53
02	14.6	913	913	828	40	45
062-BROMOFORM						
01	68.0	909	909	791	55	63
02	12.6	908	908	787	54	67
063-METHYLENE CHLORIDE						
01	46.7	913	913	819	41	53
02	10.3	910	910	761	58	91
064-CHLOROBENZENE						
01	68.1	920	919	861	21	37
02	17.7	918	918	876	13	29
VOLATILE AROMATICS IN MICROGRAMS/LITER						
065-BENZENE						
01	55.9	990	990	890	39	61
02	9.30	990	990	897	37	56
066-ETHYLBENZENE						
01	56.4	991	991	913	33	45
02	10.4	993	993	887	40	66
067-TOLUENE						
01	44.7	990	990	916	25	49
02	7.60	991	991	880	40	71
094-1,2-DICHLOROBENZENE						
01	52.0	955	955	832	55	68
02	11.7	955	955	883	32	40

Summary Report for All Participants  
USEPA Water Pollution Study WP035

Report: PE-010  
Page: 6  
Date: 16APR96

Requesting Office: ALL

Total Participants: 3,000

Sample Number	True Value	Results Reported	Usable	Accept.	Check For Error	Not Accept.
095-1,4-DICHLOROBENZENE						
01	48.3	955	955	882	39	34
02	13.4	954	954	865	36	53
096-1,3-DICHLOROBENZENE						
01	42.7	955	955	815	53	87
02	12.6	954	954	855	43	56
MISCELLANEOUS PARAMETERS						
071-TOTAL CYANIDE (IN MG/L)						
01	.0301	852	842	693	32	117
02	0.410	852	851	636	66	149
072-NON-FILTERABLE RESIDUE (IN MG/L)						
01	88.0	1,919	1,918	1,701	74	143
02	56.0	1,916	1,914	1,608	96	210
073-OIL AND GREASE (IN MG/L)						
01	46.0	1,145	1,144	976	46	122
02	18.9	1,146	1,144	893	80	171
097-TOTAL PHENOLICS (IN MG/L)						
01	2.71	707	707	627	31	49
02	1.19	711	711	643	16	52
098-TOTAL RESIDUAL CHLORINE (IN MG/L)						
01	2.80	1,488	1,482	1,135	115	232
02	0.410	1,485	1,483	1,339	43	101
Totals for WP035		150,884	150,553	128,393	7,435	14,725

\*\*\*\*\* END OF REPORT \*\*\*\*\*

Performance Evaluation Report  
USEPA Water Pollution Study WPC35

Page: 1  
Date: 16APR86

Participant ID: IL00932

Type: OTHER

Requesting Office: DCE

Sample Number	Reported Value	True Value*	Acceptance Limits	Warning Limits	Performance Evaluation
------------------	-------------------	----------------	----------------------	-------------------	---------------------------

TRACE METALS IN MICROGRAMS/LITER

09-MERCURY

01	3.18	3.10	2.03- 4.07	2.29- 3.81	Accept.
02	12.0	11.6	8.65- 14.7	9.41- 13.9	Accept.

\*\*\*\*\* END OF DATA FOR IL00932 \*\*\*\*\*

NOTE: FOR LIMITS AND TRUE VALUES, ASSUME THREE SIGNIFICANT DIGITS.

\*\*\*\*\* END OF REPORT FOR IL00932 \*\*\*\*\*

\* Based on gravimetric calculations, or a reference value when necessary.

## Silver Generation Rates from Dental Waste Water

V. Ovsey<sup>1,2\*</sup>, W. Roddy<sup>2</sup>, M. Cailas<sup>1</sup>, J Drummond<sup>1</sup>, M. Cohen<sup>2</sup>, M. Stone<sup>2</sup>, M. Babka<sup>1</sup>, R. Perry<sup>1</sup>, T. Toepke<sup>1</sup> and S. Ralls<sup>2</sup>.

University of Illinois at Chicago<sup>1</sup>, School of Public Health and College of Dentistry, and Naval Dental Research Institute<sup>2</sup>, Great Lakes, Illinois.

### ABSTRACT

Dental waste water (DWW) contains a sizeable quantity of silver from amalgam. The economic value of silver provides the impetus to recover, treat, and recycle such waste at the source. In order to quantify the silver content of the DWW, more than 70 DWW samples were collected from the Great Lakes Naval Dental Center and private dental offices. Following nitric acid digestion, silver was analyzed by the direct flame air-acetylene method for atomic absorption spectrometry. The two fractions examined were: 1) solid fraction - particles ( $\approx > 700\mu\text{m}$ ) retained on the in-line vacuum trap; 2) settled fraction - particulate settled for 24 hours. Analysis of the data identified highly skewed distributions in both fractions. The solid fraction data had a median of 414 mg Ag per day per chair and a maximum of 1,946 mg Ag per day per chair. The settled fraction had a median of 202 mg Ag per day per chair and a maximum of 1,490 mg Ag per day per chair. This data implies that the DWW can generate approximately 616 mg Ag per day per chair (sum of solid and settled fractions' medians). Predictive models were developed to describe the generation rates of silver per day from DWW. These models included as explanatory variables the number of capsules used and the dry weight of each fraction, and explained more than 78% of the variance. This initial study indicates that the amount of silver from dental waste water is significant and has great potentials for recycling.

### INTRODUCTION

Dental amalgam has been a tooth filling material for more than 150 years. Compared with other dental restorative materials, it is highly durable, relatively inexpensive and technique insensitive. Amalgam accounts for 70% to 80% of all single tooth restorations and the United States, 90 to 100 tons of amalgam are used yearly in dental restorative work (WHO, 1991). Though the composition of dental amalgam varies from manufacturer to manufacturer, the basic ingredients of amalgam are silver (20-38%), tin (11-15%), copper (0.5-25%), zinc (0.5-1%); mercury comprises (42-52%) of the total mass. In placing dental restorations a liquid waste stream that contains amalgam fragments, tooth enamel, dentine, tissues, and inorganic and organic compounds, is generated, and eventually discharged into the sewage system.

The combined discharge of this waste from many individual sources may influence the performance of the waste water treatment facilities, and their ability to meet the requirements of their National Pollution Discharge Elimination System (NPDES) permits. In recent years, many sanitary districts have had difficulties complying with the existing, or anticipated, requirements of their NPDES permits concerning priority pollutants. Unregulated small quantity generators, such as dental clinics, are likely to be identified as a possible sources causing these compliance problems. The municipality of metropolitan Seattle investigated the mercury and silver content of the waste water



generated from dental clinics (Municipality of Metropolitan Seattle, 1991). The mercury concentrations in this waste stream were found to range from 12 to 480 mg Hg/L, whereas the silver concentrations were from 12 to 62 mg Ag/L. In these cases, the mercury and silver concentrations exceeded the local discharge limits of 0.2 mg Hg/L and 3.0 mg Ag/L, respectively. However, small sample size, lack of experimental design, inability to discern between the supernatant/soluble and the particulate heavy metal content does not provide sufficient information to develop an effective treatment program. Nevertheless, these findings imply that dental offices and clinics may be discharging a significant quantity of potentially valuable silver in the form of amalgam particles in the waste water bound for treatment facilities. For example, based on this study, the Seattle Metropolitan sanitary district estimated that the 1,650 dental offices in its service region likely contribute up to 14% of the total mercury load to the local waste water treatment facilities (Municipality of Metropolitan Seattle, 1991). By extrapolation, the silver load attributable to this waste stream is likely to be approximately 10%. Similar studies in California have found comparable results (Barrucci, et. al., 1992; Rourke, 1993).

In Europe, concerns about the impact of mercury on aquatic ecosystems and the heavy metal content of compost material generated from waste water treatment sludge, resulted in mandatory treatment at the source (i.e., Switzerland, Germany, Sweden and Denmark; see Arenholt-Bindslev 1992 ). In the United States, implementation of the Great Lakes Water Quality Guidance criteria for wildlife protection, with a 1300 pg/L mercury limit and a five-year adjustment period for sanitary districts to comply with the resulting new permit requirements, will most likely speed up the requirements for mandatory treatment of this waste stream (EPA, 1995a). These conditions, an unregulated waste stream with potentially toxic and recyclable components, and a regulatory environment that is becoming more stringent, are likely to be ideal for the establishment of a successful recycling program.

To date, the recycling potentials of the dental waste water (DWW) have not been thoroughly assessed. The resulting loss in revenues and "cost" due to the resulting heavy metal load to treatment facilities remains unknown. To overcome this lack of information, a study is underway at the University of Illinois at Chicago and the Naval Dental Research Institute, Great Lakes, Illinois, which aims to characterize the properties of the DWW and identify optimum treatment methods. In this paper, results related to the silver generation rates of the DWW will be presented.

## MATERIALS AND METHODS

To date, more than 70 DWW samples have been collected. The samples had been collected from dental-chairs (i.e., units) at the clinics of the Naval Dental Center, Great Lakes, Illinois and the offices of private practitioners at the University of Illinois at Chicago, College of Dentistry. The samples generated during operations were collected in graduated clinical vacuum canisters (BEMIS System I 2,000 cc and System II 3,000 cc). Smooth walled tubing (Tygon) was used to connect these canisters to the dental units. The dental operations were recorded in detailed daily record of the number of patients seen, the number of restorative surfaces placed, the amalgam surfaces removed, the number of amalgam capsules used, performed polishing operations, and all other dental procedures that might have an influence on the properties of the DWW.

Based on the properties of the DWW and the current dental unit configuration, two components can be discerned: the first consist of solid waste material retained on the in-line vacuum trap that has a mesh size of approximately 700  $\mu\text{m}$  (Solid Fraction, see Figures 1 and 2); while the second component is a liquid heterogeneous suspension generated during dental operations, which

contains a settleable waste material portion that may be recovered through settling (Settled Fraction). Modern dental units retain these "large" particles on an in-line vacuum trap to avoid accumulation and blocking of the discharge lines. For this project, the content of this vacuum trap has been collected and analyzed. After collection, the liquid heterogeneous suspension samples were stored at approximately 4°C to prevent microbial growth and evaporation.

Each sample was processed in the laboratory no later than four weeks following collection.

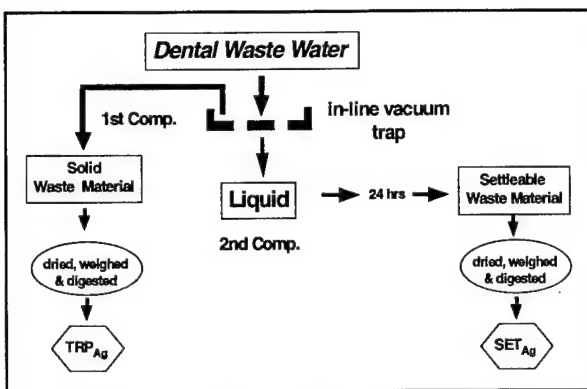


Figure 1. Schematic of the experimental design.

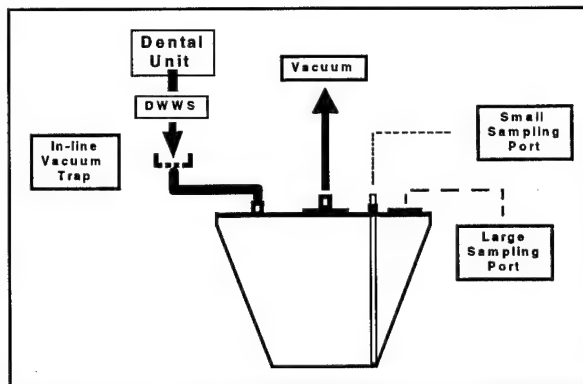


Figure 2. Schematic of the Bemis vacuum sampling canister.

Figure 1 outlines the experimental design. To quantify the components of the DWW with the most significant "reserves" of silver, two components of this waste were examined. The first consisted of solid waste material retained on the in-line vacuum trap (see Figures 1 and 2). While the second portion of the liquid component consisted of settleable waste material recovered from the liquid heterogeneous suspension of the DWW after the sample has been left to settle for 24 hours.

The large particles retained on the in-line trap were transferred to a clean, dry, pre-weighed beaker and its contents were dried to constant weight at 30°C. The dry weight of this material was determined as the difference between the total weight and the pre-weighed beaker.

Recovery of the settleable waste material was completed as follows: a suction probe was inserted in the small sampling port (i.e., 1 ml glass volumetric pipet, see Figure 2), and then the container was tipped at approximately 45 degrees to collect the settleable waste material on the bottom and the area opposite to the small sampling port (see Figure 2). In this position, the sample was left to settle for a 24 hour period under quiescent conditions. After 24 hours of settling the container was placed in an upright position, without disturbing the settled material collected on the bottom and the area opposite to the small sampling port. A one eighth ( $\frac{1}{8}$ ) ID tubing was attached to the suction probe (Tygon tubing). A 50-ml syringe was used to create a vacuum in the tubing to syphon off the supernatant into a separate clean dry container.

After the separation of the supernatant, the settled waste material was transferred into a clean, dry, pre-weighed beaker. Any solid residue that remained attached to the container was rinsed away by using double deionized (DDI) water. After the rinse and transfer of all remaining waste material into the beaker, the beaker content was dried to constant weight at 30°C. The dry weight of this material was determined as the difference between the total weight and the pre-weighed beaker. The silver has to be released from the dental amalgam (waste material) into aqueous solution, which can then be analyzed. This was accomplished by dissolving the waste material with an appropriately selected digestion procedure. After reviewing the relevant literature (Cotton and Wilkinson, 1966;

Fischer and Peters, 1970; Guenther, 1975), the nitric acid digestion method recommended by the APHA (1992) was selected (SM 3030 E. - Nitric Acid Digestion). The digested sample was cooled and transferred into a "Class A" volumetric flask, then diluted to the mark with DDI water, and transferred into a light absorbing 250 or 500 ml polypropylene container, and stored at 4°C.

A Perkin-Elmer 5000 Atomic Absorption Spectrometer (AA) was used for silver determination by the direct flame air-acetylene method (APHA, et. al., 1992; and Perkin-Elmer, 1982). Stringent quality control (QC) procedures were applied to ensure precision and accuracy of the analyses (EPA, 1979; APHA, et. al., 1992; EPA; 1995b). All statistical data analyses and model development were performed using the Statistical Application System (SAS), release 6.10.

## RESULTS AND DISCUSSION

The descriptive statistics for the variables describing the silver content of the DWW are presented in Table 1. To simplify the presentation, the silver content of the waste material retained on the in-line trap is designated as  $TRP_{Ag}$ ; whereas, the silver content of the settleable material as  $SET_{Ag}$  (see Figure 1). The histograms for these observations are presented in Figures 3 and 4. As seen from the histograms and the descriptive statistics, both distributions are highly skewed. The median generation rate from DWW of 0.414 g Ag per day per unit was found for the  $TRP_{Ag}$ , while 0.202 g Ag per day per unit was identified for the  $SET_{Ag}$ .

The adequacy of the DWW sample size, which has been used in the estimation of generation rates was examined as well. For this

purpose the mean, median, and 25% upper quartile statistics of the  $TRP_{Ag}$  and  $SET_{Ag}$  variables were used. The sequential estimate of these statistics, facilitated the identification of a stabilization range beyond which the analysis of additional DWW samples does not improve significantly the generation estimates. In Figures 5 and 6 the sequentially estimated statistics are presented as a function of the sample size. As seen from these figures 50 DWW samples are

Parameters per Dental Unit	$TRP_{Ag}$	$SET_{Ag}$
N (observations)	57	66
Mean (mg Ag/day)	589	428
Median (mg Ag/day)	414	202
Standard Deviation (mg Ag/day)	534	437
Skewness	1.01	0.99
25% Upper Quartile (mg Ag/day)	924	642
Maximum (mg Ag/day)	1946	1490

Table 1. Descriptive statistics for the silver content of the DWW

sufficient for deriving relatively stable estimates for silver generation, since the addition of more samples did not change significantly the properties of the mean, median, and 25% upper quartile.

Variables such as the dry weight of the solid waste material retained in the in-line vacuum trap ( $TRP_{WT}$ ), the dry weight settleable waste material ( $SET_{WT}$ ), the number of amalgam surfaces placed, the surfaces of amalgam removed, the number of consumed amalgam capsules, and the volume of waste water generated, are likely to have a significant influence on the amount of generated waste.

Therefore, they have been selected as explanatory variables for model development. The descriptive statistics of these variables are presented in Table 2. As seen from this table, the volume of generated waste water is highly skewed with a median value of approximately 312 ml per day per dental unit.

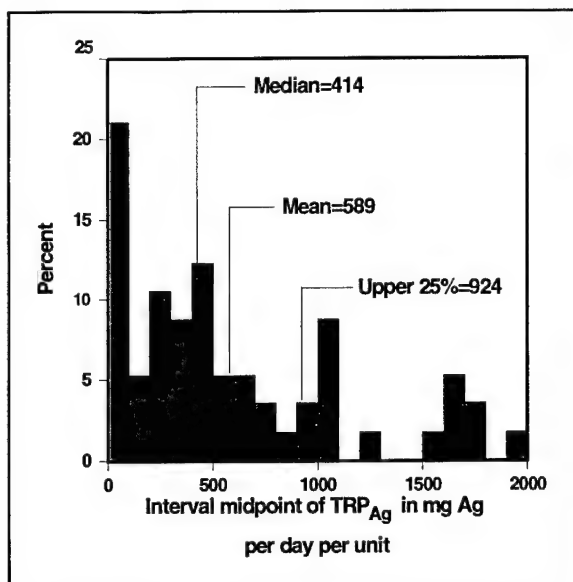


Figure 3. Histogram of the silver content of the Solid Waste Material ( $TRP_{Ag}$ ).

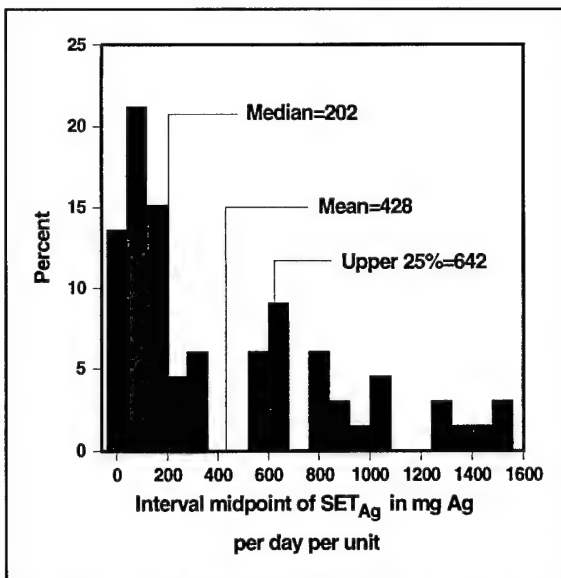


Figure 4. Histogram of the silver content of the Settleable Waste Material ( $SET_{Ag}$ ).

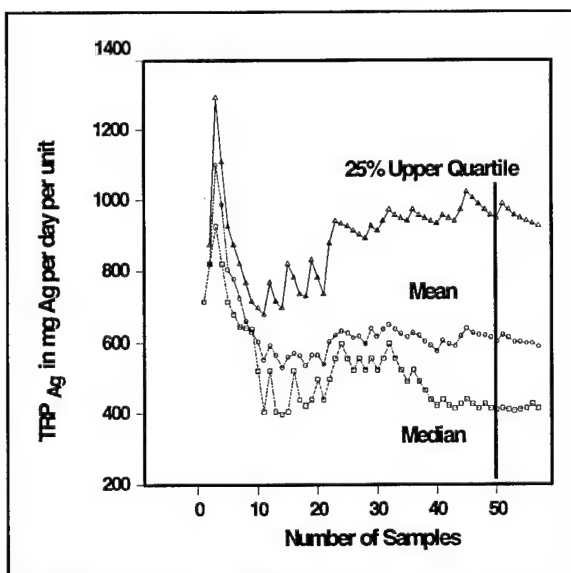


Figure 5. Sequential estimation of the mean, median, and 25% upper quartile for the silver content of the Solid Waste Material ( $TRP_{Ag}$ ).

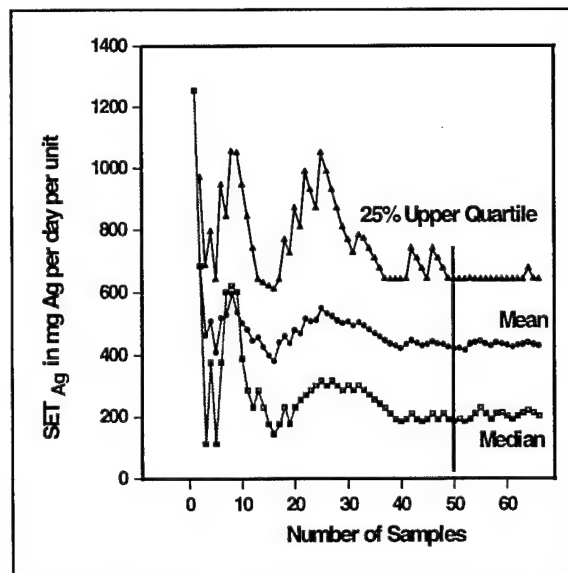


Figure 6. Sequential estimation of the mean, median, and 25% upper quartile for the silver content of the Settleable Waste Material ( $SET_{Ag}$ ).

At the conception of this study, a relationship between silver generation rates and procedures & measures were postulated. The purpose of developing predictive models is to quantify the recycling potential of silver in the dental waste water. The generation rates of silver in solid and settleable waste materials of the DWW were patterned opposite the independent variable(s).

The relationships between the explanatory variables and the silver generation rates have been examined with the application of the Spearman correlation coefficient (Sprent, 1989). The correlation coefficient estimates [i.e.,  $\rho$  (Rho)] are given in Table 3. Following  $\rho$ , the probability

value is given. Under the null hypothesis  $\rho$  is equal to zero [i.e., Probability  $> |\rho|$  under  $H_0$ :  $\rho=0$  (e.g.,  $\rho=0$  is not significance, while  $\rho=\pm 1$  is highly significant)].

The sample size used to estimate the coefficient is below the probability value. From this table, the silver generation variables are highly correlated with the dry weight variables ( $TRP_{WT}$  and  $SET_{WT}$ ). In addition, operational parameters such as the surfaces-placed and the number of amalgam-capsules, are significantly correlated with the silver generation variables (i.e., even at a 0.01% level). Volume, and some of other operational parameters such as the number of patients, the number of surfaces removed, do not suggest a significant association.

A graphical depictions of the silver generation variables as a function of explanatory variables (i.e.,  $TRP_{Ag}$  vs.  $TRP_{WT}$  or consumed capsules, and  $SET_{Ag}$  vs.  $SET_{WT}$  or consumed capsules) are presented in Figures 7 through 10. These figures include model regression lines and the 95% prediction limits. These figures show a distinct linear pattern between silver generation as a function of the predictor variables. Based on these findings, three linear regression models were selected for each silver generation variable (i.e.,  $TRP_{Ag}$  and  $SET_{Ag}$ ). The dry weight variables (see note A in Table 4) and the consumed capsules (see note B in Table 4) were used as predictor variables. The parameter estimates for these models and basic diagnostic statistics (performance statistics) are presented in Table 4. The number of surfaces placed was excluded from the model since its parameter estimate was not significant at a 5% level.

Parameters	Explanatory variables (per day per dental unit)					
	$TRP_{WT}$ (g)	$SET_{WT}$ (g)	Surfaces removed	Surfaces placed	Amalgam capsules	Volume (ml)
N	57	66	59	61	57	68
Mean	1.81	1.90	1.7	7.6	9.8	437.0
Median	1.38	1.39	1.0	5.0	5.0	312.5
Standard Deviation	1.54	1.48	2.0	7.0	11.0	416.0
Skewness	1.14	1.05	1.35	1.64	1.83	2.30
25% upper quartile	2.56	2.67	2.25	11.0	12.0	601.3
Maximum	6.13	5.77	8	33	48	2150

Table 2. Descriptive statistics for the explanatory variables.

	Explanatory variables (per day per dental unit)					
	$TRP_{WT}$ (g)	$SET_{WT}$ (g)	Surfaces removed	Surfaces placed	Amalgam capsules	Volume (ml)
$TRP_{Ag}$ (mg/day)	0.97714 0.0001 57	0.76563 0.0001 55	0.19141 0.1785 51	0.82560 0.0001 51	0.82253 0.0001 48	-0.00710 0.9582 57
$SET_{Ag}$ (mg/day)	0.72617 0.0001 55	0.89203 0.0001 66	0.12096 0.3701 57	0.71337 0.0001 59	0.75055 0.0001 55	0.07794 0.5339 66

Table 3. Spearman correlation coefficients between silver generation and explanatory variables.

Dependent variable	Explanatory variables			Performance statistics			Model
	Intercept	A Weight	B Amalgam capsules	F-value	R <sup>2</sup>	F	
$TRP_{Ag}$	-13.9	333.1 <sup>C</sup>	—	689.7 <sup>C</sup>	0.932	1,304,461	1
	125.2 <sup>E</sup>	—	42.3 <sup>C</sup>	182.0 <sup>C</sup>	0.798	3,289,220	2
	19.9	255.8 <sup>C</sup>	126 <sup>C</sup>	478.3 <sup>C</sup>	0.955	964,405	3
$SET_{Ag}$	-95.3 <sup>E</sup>	275.8 <sup>C</sup>	—	426.4 <sup>C</sup>	0.869	1,755,467	4
	39.3	—	34.9 <sup>C</sup>	192.3 <sup>C</sup>	0.785	2,451,106	5
	-93.4 <sup>E</sup>	201.2 <sup>C</sup>	126 <sup>D</sup>	219.9 <sup>C</sup>	0.894	1,343,681	6

A - refers to  $TRP_{WT}$  for  $TRP_{Ag}$  and  $SET_{WT}$  for  $SET_{Ag}$   
B - all capsules were double capsules.  
C - indicates significant model or parameter at 0.01% level (F-value and t-test statistic).  
D - indicates significant parameter at less than 0.1% level (t-test statistic).  
E - indicates significant parameter at less than 2.0% level (t-test statistic).  
F - selection of most appropriate model based on the smallest FRESS statistic value (potentially useful for discriminating between alternative models).

Table 4. Predictive models for silver generation

The number of surfaces placed was excluded from the model since its parameter estimate was not significant at a 5% level.

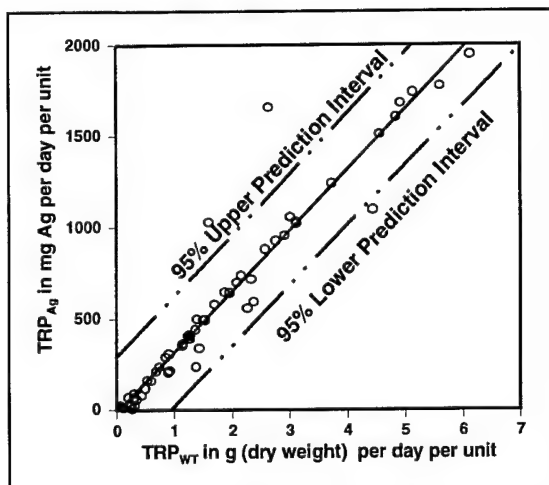


Figure 7. The silver content of the Solid Waste Material ( $TRP_{Ag}$ ) as a function of the dry weight of the Solid Waste Material ( $TRP_{WT}$ ).

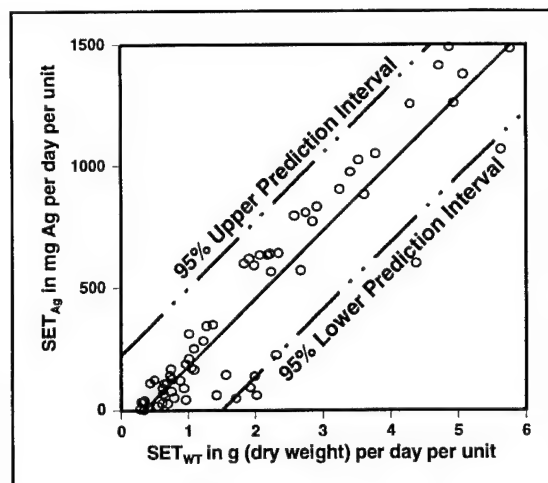


Figure 8. The silver content of the Settleable Waste Material ( $SET_{Ag}$ ) as a function of the dry weight of the Settleable Waste Material ( $SET_{WT}$ ).

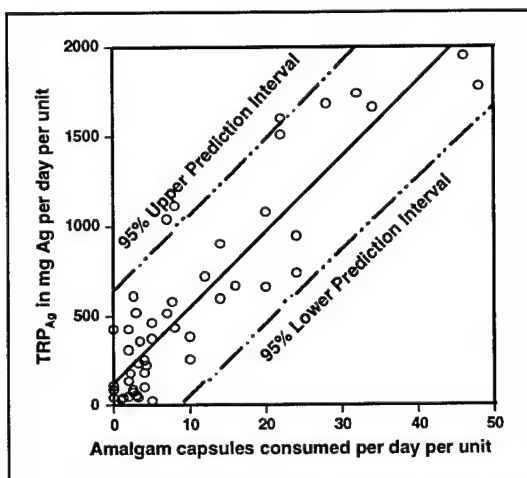


Figure 9. The silver content of the Solid Waste Material ( $TRP_{Ag}$ ) as a function of amalgam capsules consumed.

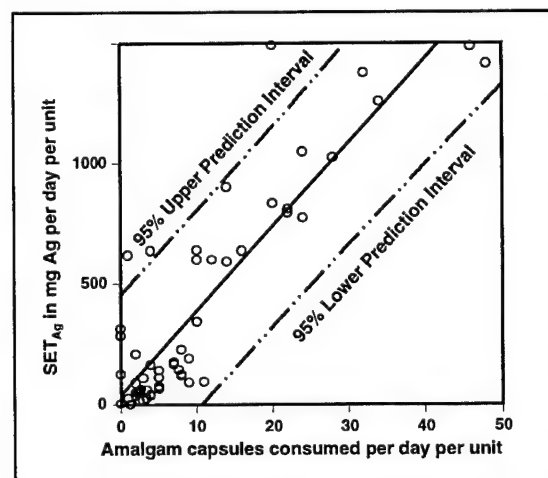


Figure 10. The silver content of the Settleable Waste Material ( $SET_{Ag}$ ) as a function of amalgam capsules consumed.

The best model structure for predicting the silver retained in the in-line trap (i.e.,  $TRP_{Ag}$ ), should contain both of the predictor variables (i.e.,  $TRP_{WT}$  and the number of consumed capsules; see model 3 in Table 4, and the graphical depiction of the prediction surface in Figure 11). This selection is justified from the model performance statistics found in Table 4, since the: F-test for regression is significant,  $R^2$  is relatively high, and the PRESS statistic has the lowest value. Further residual analysis confirmed these findings. The selected model should serve two purposes: a) facilitate recyclers in predicting the amount of recoverable silver from the waste material retained on the in-line vacuum trap, and b) serve as a tool for performing cost benefit analyses and pollution prevention planning. By considering these two model objectives, model (1) will apparently be more appropriate for recycling purposes, since it requires only the dry weight of the waste material. For in house



pollution prevention planning, model (2) may be the most appropriate, since clinics maintain an inventory of the consumed amalgam capsules. Both these models, (1) and (2) perform relatively well in predicting the amount of generated silver. For silver generated in the settleable waste material similar conclusions were drawn (see Table 4). Figure 12, presents similar results as well. Conceptually these findings are justified since the two silver generation variables are highly correlated (i.e., Spearman correlation coefficient of 0.78).

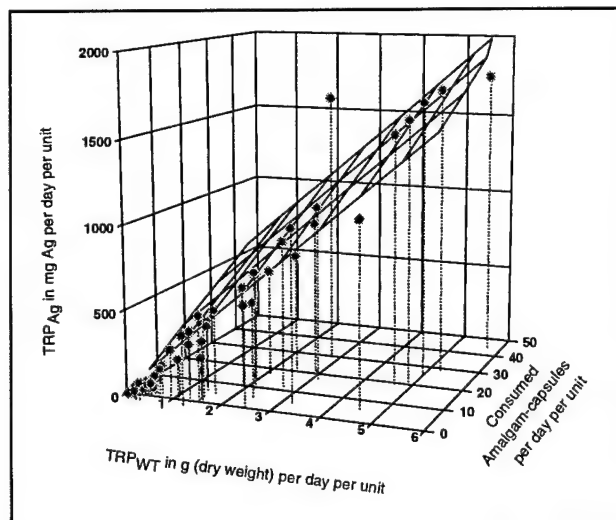


Figure 11. The graphical depiction of the prediction surface of the Solid Waste Material's silver content ( $TRP_{Ag}$ ), dry weight ( $TRP_{WT}$ ) and the number of consumed amalgam capsules.

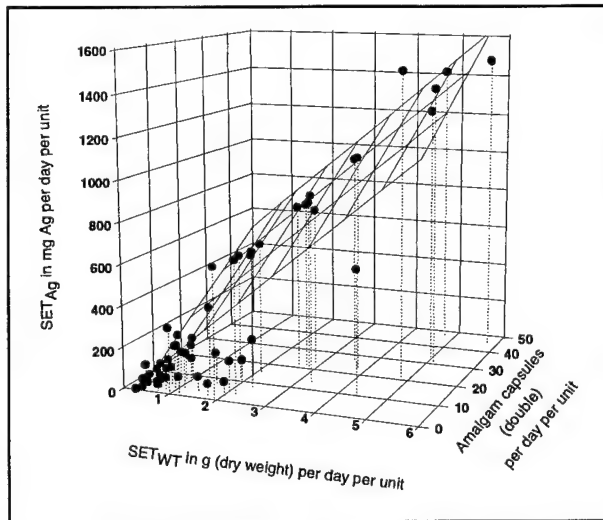


Figure 12. The graphical depiction of the prediction surface of the Settleable Waste Material's silver content ( $SET_{Ag}$ ), dry weight ( $SET_{WT}$ ) and the number of consumed amalgam capsules.

## CONCLUSIONS

The results from this study illustrate that the dental waste water has a relatively high content of silver, which may be a potential revenue source if recycled. To quantify the silver content of the DWW two components of this waste have been measured: the first consist of solid waste material retained on the in-line vacuum trap that has a mesh size of approximately  $700\ \mu\text{m}$ ; while the second component was the liquid heterogeneous suspension generated during dental operations, which contain a settleable waste material portion that may be recovered through settling.

A conservative estimate of the generation rate of settleable silver from individual dental units is approximately 202 mg of silver per day with a 25% quartile, which can reach 642 mg of silver per day. This amount of silver is likely to be discharged into the sewage lines. The quantity of silver retained on the in-line trap was almost double the settleable silver, with a conservative estimate for this rate of 414 mg of silver per day. The silver retained in the in-line trap had a 25% quartile of 924 mg of silver per day.

From the results of this study, predictive models of silver generation have been developed. Based on practical considerations a model is proposed for predicting the amount of solid waste

material retained on the in-line vacuum trap based on the dry weight of this component (i.e.,  $TRP_{Ag}$  vs.  $TRP_{WT}$ ). This model is appropriate for precious metal recyclers, since they can estimate the silver quantity based only on the dry weight of the waste material. For in house recycling planning, the predictive model based on the number of consumed capsules is more appropriate, since clinics maintain an inventory of these amalgam capsules. Similar models have been developed for the settleable waste material portion.

### ACNOWLEDGEMENTS

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# Antibacterial Activity of Dentin Bonding Systems, Resin-modified Glass Ionomers, and Polyacid-modified Composite Resins

J C MEIERS • G A MILLER

## Clinical Relevance

The antibacterial activity of the various primers was unexpected and indicates a dual antibacterial action of these systems.

## SUMMARY

The antibacterial effects of the dentin bonding systems Syntac, ProBOND, Gluma 3-Step, the resin-modified glass ionomers Photac-Fil, Fuji Lining LC, Fuji II LC, and the polyacid-modified composite resins VariGlass, Geristore, and Infinity were evaluated using the cariogenic bacteria *S mutans*, *L salivarius*, *S sobrinus*, and *A viscosus* in vitro with a modified cylinder drop plate agar diffusion assay. All glass ionomers, the polyacid-modified composites, and the primers and adhesives of the dentin bonding systems exhibited various degrees of antibacterial activity against most of the test bacteria. The antibacterial activity of the adhesives of dentin bonding systems was anticipated because of the glutaraldehyde used in their formulations. However, the antibacterial activity of the various primers was unexpected and indicates a dual antibacterial action of these systems.

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## INTRODUCTION

Bacteria have been implicated in pulpal inflammation after restoration placement (Brännström, 1984; Bergenholtz & others, 1982; Cox, 1992). The source of bacteria may be from their incomplete removal during cavity preparation or from their reappearance due to microleakage at the margins of the restoration (Going, 1972; Mejare, Mejare & Edwardsson, 1979; Bergenholtz & others, 1982; Brännström, 1984; Ben Amar, 1989; Cox, 1992).

Currently, there are no universally accepted objective tests to determine the bacterial status of a preparation during caries removal. Traditionally, the effectiveness of caries removal has been a subjective judgment based on the color and texture of dentin in the cavity preparation—which may or may not accurately reflect the actual bacterial status (Fisher, 1981; Baum, Phillips & Lund, 1985; Kidd, Joyston-Bechal & Beighton, 1993b). Caries-disclosing solutions of either 0.5% basic fuchsin or 1.0% acid red have been proposed as an objective method for determining the bacterial status of dentin (Sato & Fusayama, 1976; Fusayama, Takatsu & Itoh, 1979; Franco & Kelsey, 1981; Anderson & Charbeneau, 1985; Anderson, Loesche & Charbeneau, 1985; Kidd & others, 1989). However, there is growing skepticism about the accuracy of these dyes in reflecting the true bacterial condition of dentin (Kidd, Joyston-Bechal & Beighton, 1993a; Boston & Graver, 1994; Yip, Stevenson & Beeley, 1994).

Secondary caries, whether the result of bacterial invasion through microleakage or from residual bacteria left in the cavity preparation, has consistently been found to be the most common reason for replacement of amalgam (Mjör, 1981; Boyd & Richardson, 1985; Klausner & Charbeneau, 1985; Klausner, Green & Charbeneau, 1987; Qvist, Qvist & Mjör, 1990a; York & Arthur, 1993) and composite restorations (Qvist, Qvist & Mjör, 1990b; York & Arthur, 1993).

A possible solution to this problem would be the use of dental materials that are antibacterial. Glass ionomers, various dentin bonding systems, and calcium hydroxide have been shown in vitro to be antibacterial toward various types of oral bacteria (Forsten & Soderling, 1974; Barkhordar & Kempler, 1989; Barkhordar & others, 1989; DeSchepper, White & von der Lehr, 1989; Scherer, Lippman & Kaim, 1989; Scherer, Cooper & Antonelli, 1990; Prati & others, 1993; Loyola-Rodríguez, García-Godoy & Lindquist, 1994). Additionally, calcium hydroxide preparations and a glutaraldehyde-containing dentin bonding agent have been shown to reduce or eliminate bacteria

within a preparation in vivo (Leung, Loesche & Charbeneau, 1980; Fairbourn, Charbeneau & Loesche, 1980; Felton, Bergenholtz & Cox, 1989).

The continuing emergence of newly formulated restorative materials, which often replace previous materials whose antibacterial properties may be known, requires their evaluation for antibacterial properties to update those clinicians who may want to employ them as a method for controlling bacterial levels within the tooth during and after restorative procedures. The purpose of this study is to examine the antibacterial activity of three current dentin bonding systems containing glutaraldehyde, three resin-modified glass-ionomer cements, and three polyacid-modified composite resins against the cariogenic bacteria *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus salivarius*, and *Actinomyces viscosus* using a modified cylinder drop plate agar diffusion assay.

## METHODS AND MATERIALS

The dental materials evaluated in this study are shown in Table 1. The antibacterial activity of each material was evaluated against the following bacteria: *Lactobacillus salivarius* subsp *salivarius* (ATCC# 11741); *Streptococcus sobrinus* (ATCC# 33478); *Actinomyces viscosus* (ATCC# 15987), and *Streptococcus mutans* (ATCC# 25175), using a modified cylinder drop plate agar diffusion assay system.

A 10 µl inoculating loop of each bacteria was obtained from trypticase soy agar slants (Difco, Labonia, MI 48152) of the original ATCC cultures that had been incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air for 48 hours. The bacteria were placed in duplicate 10 ml tryptic soy broth (Difco) cultures at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air for 48 hours. Broth cultures for each bacteria were added aseptically to separate 1 liter flasks of sterile tryptic soy broth. Each flask was incubated in a 37 °C shaker water bath for 72 hours. Each bulk culture was then placed into 500 ml centrifuge bottles (DuPont Sorvall Centrifuge Products, Wilmington, DE 19898), spun at 10,000 rpm (16,300 rcf) for 10 minutes using a GSA rotor in a Sorvall Superspeed Centrifuge. Pellets for each bacteria were pooled and resuspended in 50 ml of tryptic soy broth.

Table 1. Dental Materials Used in Study

Materials	Components Tested	Manufacturer
<b>Dentin Bonding Systems</b>		
Syntac	primer, adhesive, adhesive without glutaraldehyde, Heliobond resin	Vivadent USA, Amherst, NY 14228
Gluma 3-Step	conditioner, primer, sealer	Miles Inc, South Bend, IN 46614
ProBOND	primer, adhesive, adhesive without glutaraldehyde	L D Caulk/Dentsply, Milford, DE 19963
<b>Resin-modified Glass-Ionomer Cements</b>		
Fuji Lining LC	complete cement (powder + liquid) & liquid only	GC America, Chicago, IL 60658
Fuji II LC	complete cement (powder + liquid)	GC America
Photac-Bond	complete cement (powder + liquid)	ESPE/Premier, Norristown, PA 19401
<b>Conventional Glass-Ionomer Cement</b>		
Ketac-Bond	complete cement (powder + liquid)	ESPE/Premier
<b>Polyacid-modified Composite Resins</b>		
VariGlass	complete mix (powder + liquid)	L D Caulk/Dentsply
Geristore	complete mix (paste + paste)	Den-Mat Corp, Santa Maria, CA 93456
Infinity	complete mix (paste + paste)	Den-Mat Corp

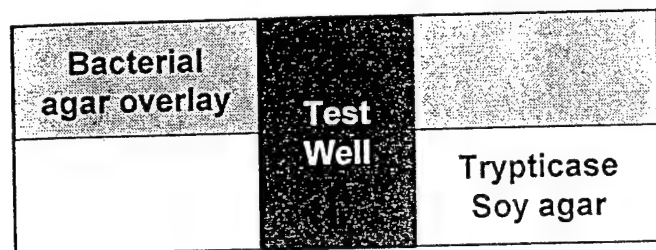


Figure 1. Cross section of modified cylinder drop plate agar design

Each bacterial suspension was mixed with 2 liters of sterile trypticase soy agar that had been cooled to 50 °C. Ten milliliters of the resultant suspension was overlaid onto trypticase soy agar plates (NIH Media Bank, Bethesda, MD 20892) that contained 15 ml of agar per plate (Figure 1). Once the overlay solidified, wells of 4.5 mm in diameter were created in the agar with the large bore end of a sterile Pasteur pipet (Ladd Research Industries, Burlington, VT 05402) (Figure 2). There were no more than six wells per plate.

The wells were then filled to the rim with either 1.0% glutaraldehyde (from 70% EM GRADE, Polysciences, Inc, Warrington, PA 18976), which acted as a control and was present in each plate, or in the material to be evaluated. Syntac and ProBOND additionally had adhesives tested that were identical in chemistry to those normally contained in their commercially available dentin bond systems but without glutaraldehyde. The components of each dentin bonding system that required visible light activation and all the resin-modified glass ionomers and the polyacid-modified composite resins were irradiated for 40 seconds with a visible light curing

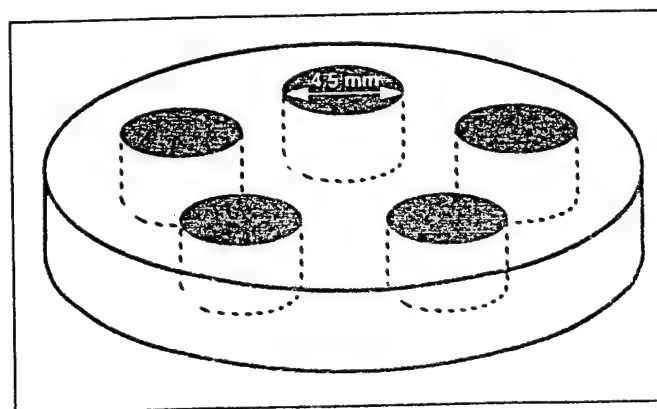


Figure 2. Layout of typical petri dish for agar diffusion test

unit (L D Caulk/Dentsply, Milford, DE 19963) immediately after placement into the agar wells. This light unit was tested for adequate light output before each session using a curing radiometer (Demetron Research Corp, Danbury, CT 06810). All materials were handled under aseptic conditions and mixed according to the manufacturer's instructions. Each dental material evaluation was repeated five times.

All plates were incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub>, 95% air for 24 hours. Zones of bacterial growth inhibition were measured to the nearest hundredth of a millimeter using a dial caliper (Fowler, Munich, Germany). All measurements of zone diameter included the diameter of the well and were measured at the widest part of the zone (Figures 3 and 4).

pH determinations of the primers, adhesives, and resins of the three dentin bonding systems were

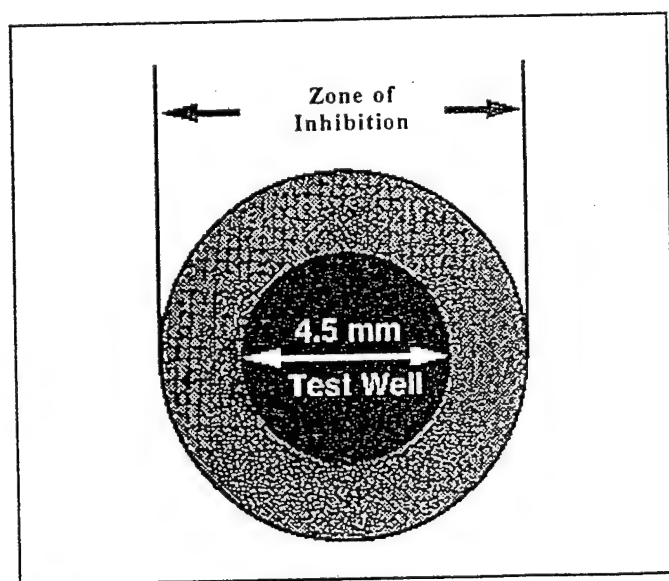


Figure 3. How the measurements on the zones of inhibition were taken

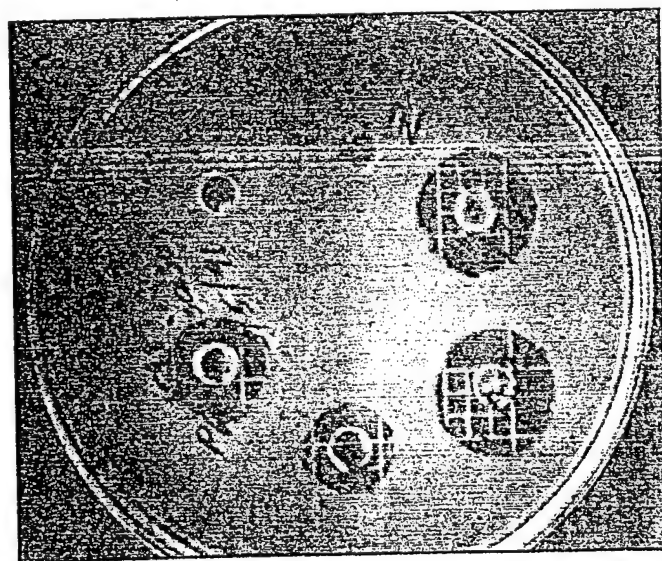


Figure 4. Actual agar diffusion test showing both lytic zones of inhibition (halo) and nonlytic, no-response zones

Table 2. Antimicrobial Activity--Syntac

Organism	Component				
	Control (1% glutaraldehyde)	Primer	Adhesive without glutaraldehyde	Adhesive	Heliobond Resin
<i>S mutans</i>	11.86 ± 0.3	20.02 ± 2.5	0	13.72 ± 0.8	0
<i>S sobrinus</i>	13.31 ± 0.3	18.38 ± 0.3	0	17.33 ± 0.8	0
<i>A viscosus</i>	9.44 ± 0.6	28.03 ± 2.1	0	13.12 ± 1.9	0
<i>L salivarius</i>	9.35 ± 0.4	16.51 ± 1.3	0	11.89 ± 1.2	0

Zones of inhibition (mm); n = 5; Mean ± SD.

performed using an Orion EA 940 expandable ionanalyzer (Orion Research, Boston, MA 02129) after the pH electrode (Orion Research) was calibrated with standard buffer solutions at pH 4.01 and 7.00.

Zones of inhibition data for the test bacteria were analyzed using ANOVA and Student-Newman-Keuls

Table 3. Antimicrobial Activity--Gluma 3-Step Bonding System

Organism	Component			
	Control (1% glutaraldehyde)	Conditioner	Primer	Sealer
<i>S mutans</i>	11.25 ± 0.3	14.20 ± 1.7	15.24 ± 0.8	0
<i>S sobrinus</i>	13.54 ± 0.3	18.08 ± 0.8	21.45 ± 0.6	0
<i>A viscosus</i>	10.05 ± 0.2	14.66 ± 1.5	15.24 ± 0.3	0
<i>L salivarius</i>	10.03 ± 0.2	14.10 ± 1.2	15.01 ± 0.7	0

Zones of inhibition (mm); n = 5; Mean ± SD.

multiple comparison test within each dental material at a significance level of  $P < 0.05$ . The lack of information on the agar diffusion coefficients of the various dental materials and the strong suspicion that these various dental materials would possess different agar diffusion coefficients would make statistical comparisons between materials imprecise and therefore were not performed.

## RESULTS

The antibacterial effects of the various dental materials are shown in Tables 2-6. Tables 2-4 show the zones of inhibition produced against the test

bacteria by the various components of the three dentin bonding systems. There was no consistent ranking regarding the susceptibility of the test bacteria to the conditioner, primers, or adhesives of the dentin bonding systems for the three systems.

Syntac primer produced its greatest inhibitory effect against *A viscosus*, while its adhesive component was most effective against *S sobrinus* (Table 2). The adhesive without the glutaraldehyde and Heliobond resin

displayed no inhibitory effect.

Gluma 3-Step Bonding System conditioner and primer both showed significantly greater inhibition against *S sobrinus* than the other three test bacteria (Table 3). The sealer component showed no inhibitory action.

ProBOND primer had its greatest inhibitory effect on *A viscosus*, while the adhesive had its greatest effect on both *A viscosus* and *S sobrinus* (Table 4). The adhesive without the glutaraldehyde showed no inhibitory effect.

The resin-modified glass ionomers all produced varying degrees of inhibitory effects on the test bacteria (Table 5). *Streptococcus mutans* displayed a significantly greater inhibition by Fuji II LC, Fuji Lining LC, and the conventional glass ionomer Ketac-Fil than did *S sobrinus*, *A viscosus*, or *L salivarius*. The liquid component of Fuji Lining LC, when compared to the complete cement, had a significantly greater inhibitory effect on each of the test bacteria.

The inhibitory effect of the polyacid-modified composite resins are shown in Table 6. Infinity and Geristore inhibited *L salivarius* and *S mutans* but did not inhibit *S sobrinus* or *A viscosus*. VariGlass

Table 4. Antimicrobial Activity--ProBOND

Organism	Component			
	Control (1% glutaraldehyde)	Primer	Adhesive without glutaraldehyde	Adhesive
<i>S mutans</i>	11.10 ± 0.2	9.68 ± 0.3	0	9.86 ± 0.7
<i>S sobrinus</i>	13.80 ± 0.3	14.77 ± 0.6	0	13.34 ± 0.9
<i>A viscosus</i>	9.40 ± 0.3	17.62 ± 2.3	0	14.45 ± 1.3
<i>L salivarius</i>	9.86 ± 0.1	11.18 ± 1.6	0	6.51 ± 0.2

Zones of inhibition (mm); n = 5; Mean ± SD.

Table 5. Antimicrobial Activity—Resin-modified Glass Ionomers/Conventional Glass Ionomers

Organism	Product				
	Control (1% glutaraldehyde)	Fuji II LC	Fuji Lining LC	Photac-Bond	Ketac-Bond
<i>S mutans</i>	11.36 ± 0.4	18.43 ± 1.7	20.00 ± 2.0 (L-37.13 ± 0.5)	11.21 ± 0.1	15.44 ± 1.1
<i>S sobrinus</i>	13.65 ± 0.2	12.03 ± 0.3	16.17 ± 0.3 (L-39.23 ± 2.0)	10.34 ± 2.1	13.46 ± 0.2
<i>A viscosus</i>	9.06 ± 0.4	6.80 ± 0.2	15.40 ± 1.2 (L-32.47 ± 1.7)	11.25 ± 0.4	13.16 ± 1.4
<i>L salivarius</i>	9.99 ± 0.2	9.39 ± 0.4	9.29 ± 0.4 (L-22.91 ± 1.0)	8.50 ± 0.2	6.38 ± 0.2

Zones of inhibition (mm); n = 5; Mean ± SD; L = liquid only, no powder added.

was inhibitory to all test bacteria, with *S sobrinus* and *A viscosus* showing significantly greater sensitivity than *S mutans* or *S sobrinus*.

Table 7 is a summary of the antibacterial activity of the various dental materials that allow inter-material comparison. It is arranged to display whether there was or was not an inhibitory action present.

The pH values and general composition of the various components of Syntac, Gluma 3-Step, and ProBOND, and the liquid of Fuji Lining LC are shown in Table 8. The primers for Syntac and ProBOND and the conditioner for Gluma 3-Step all had pH's below 4. Heliobond resin, the adhesives for ProBOND and Syntac, and the sealer for Gluma 3-Step had pH's of 4.6 or higher.

## DISCUSSION

The results of this study indicate that the dentin bonding systems and the resin-modified glass ionomers were inhibitory against all the test bacteria, while two of the three polyacid-modified composite resins were not. This confirms and extends the knowledge base gathered from previous investigations on the bacterial effects of other types of glass ionomers and dentin bonding systems (DeSchepper & others, 1989; Loyola-Rodriguez & others, 1994; Barkhordar & others, 1989; Scherer & others, 1989; Emilson & Bergenholtz, 1993; Prati & others, 1990; Scherer & others, 1990). The polyacid-modified composite resin materials Infinity and Geristore had no previous published data regarding their bacterial inhibitory effects, and VariGlass had not been tested against all the bacteria in this study.

The inhibitory effects produced by Syntac adhesive, ProBOND adhesive, and Gluma 3-Step primer were anticipated because they contained glutaraldehyde in their composition. ProBOND and Gluma 3-Step are newer and slightly modified versions of similar products that have been previously shown to have bacterial inhibitory effects (Scherer & others, 1990; Felton & others, 1989). These were chosen for use in our study as standards against which the most recent glutaraldehyde-containing dentin

bonding system, Syntac, could be measured. However, what was unanticipated was the presence of antimicrobial activity in the primers of Syntac and ProBOND, as well as the cleanser of Gluma 3-Step. Emilson and Bergenholtz (1993) found similar results with some of the dentin bonding systems they were investigating. However, they found that this effect was material specific and not consistent across all dentin bonding systems tested.

The antibacterial effects shown by the primers/cleaners in this study may be related to either their pH or chemical composition. The pH's of all the primers/cleaners were less than 4, and this pH or less is reported to be bacteriocidal (Davis & others, 1967). The pH within the agar surrounding the wells was not measured, but if these solutions did drop the pH in the agar below 4, this could explain their effect. Irrespective of the exact nature of this result, it is evident that these dentin bond systems possess antimicrobial effects during several aspects of their application.

Table 6. Antimicrobial Activity—Polyacid-modified Composite Resins

Organism	Product			
	Control (1% glutaraldehyde)	Infinity	Geristore	VariGlass
<i>S mutans</i>	11.17 ± 0.2	6.48 ± 0.5	7.28 ± 0.2	14.52 ± 0.6
<i>S sobrinus</i>	13.59 ± 0.2	0	0	25.02 ± 2.7
<i>V viscosus</i>	10.07 ± 0.2	0	0	21.50 ± 1.3
<i>L salivarius</i>	10.04 ± 0.5	7.90 ± 0.2	8.82 ± 0.4	12.66 ± 0.6

Zones of inhibition (mm); n = 5; Mean ± SD.



Table 7. Inter-Material Antibacterial Activity Summary

MATERIAL	TEST BACTERIA			
	<i>S mutans</i>	<i>S sobrinus</i>	<i>A viscosus</i>	<i>L salivarius</i>
Syntac				
Primer	+	+	+	+
Adhesive	+	+	+	+
Resin	-	-	-	-
Gluma 3-Step				
Conditioner	+	+	+	+
Primer	+	+	+	+
Sealer	-	-	-	-
ProBOND				
Primer	+	+	+	+
Adhesive	+	+	+	+
Fuji II LC	+	+	+	+
Fuji Lining LC	+	+	+	+
Photac-Bond	+	+	+	+
Ketac-Bond	+	+	+	+
Infinity	+	-	-	+
Geristore	+	-	-	+
VariGlass	+	+	+	+

+ = positive inhibitory reaction.  
 - = no inhibitory reaction.

The bacterial inhibition displayed by our group of resin-modified glass ionomers reconfirms a property for this category of dental materials that previous investigators had discovered when testing other resin-modified glass-ionomer cements (Scherer & others, 1990; Loyola-Rodríguez & others, 1994). Ketac-Bond was included in the analysis with the resin-modified glass ionomers as a standard of comparison, since it had been shown by other investigators to be inhibitory to the same or similar types of bacteria used in our study (Scherer & others, 1989; Barkhordar & others, 1989; Loyola-Rodríguez & others, 1994).

The antibacterial properties of glass-ionomer cements have been related to either their low initial pH, fluoride release, or other chemical constituents found within the powder (Scherer & others, 1989; DeSchepper & others, 1989; Loyola-Rodríguez & others, 1994). We tested the liquid component of Fuji Lining LC to see if it possessed any bacterial inhibitory properties on its own. Surprisingly, it showed significantly greater effect on all four bacteria than the mixed cement. This effect cannot be attributed to fluoride but could be related to its low

pH, chemical composition, or that it had a greater agar diffusion potential. This is the first reported antibacterial effect of a resin-modified glass-ionomer liquid, and it would be interesting to see if other resin-modified glass ionomers have the same inhibitory effect. DeSchepper and others (1989) reported similar antibacterial effects from some conventional glass-ionomer liquids, but all were less than that of the corresponding mixed cement.

The polyacid-modified composite resins Infinity and Geristore did not possess the same inhibitory properties against all four bacteria as seen with the dentin bonding systems or resin-modified glass ionomers. They were ineffective on *S sobrinus* and *A viscosus* but were inhibitory against *S mutans* and *L salivarius*. Why there was this difference in sensitivity between the various bacteria is not known. These are the first published data regarding these materials' antibacterial properties, and it would appear that this category of dental material may not be as effective against bacteria as conventional glass-ionomer or resin-modified glass-ionomer cements. This may be related to the group's chemical composition, which is more composite than glass ionomer in nature. Also, if fluoride release is a factor in a material's antibacterial effect, this category of material has a significantly lower fluoride release when compared to glass ionomers (Loyola-Rodríguez & others, 1994). The polyacid-modified composite resin VariGlass inhibited all four bacteria tested,

while Infinity and Geristore inhibited *S mutans* and *L salivarius* but not *S sobrinus* and *A viscosus*. VariGlass is closer in composition to that of resin-modified glass-ionomer cements, which inhibited all four bacteria in the study, than Infinity and Geristore.

Whether the antibacterial results from the materials tested in this investigation would be similar in vivo cannot be determined from this type of study. Also, the duration of this effect is not known, nor can it be hypothesized from this assay system. Is it just at the time of application, or is there a long-term effect that would be useful in case of future microleakage and bacterial infiltration? There are only a few studies that have actually verified the in vitro results with clinical testing (Leung & others, 1980; Fairbourn & others, 1980; Felton & others, 1989).

The agar diffusion test is an accepted method to initially differentiate antibacterial activity between materials; however, it has its limitations (Tobias, 1988). Without further tests, it cannot be determined whether the data gathered from a specific material reflect bacteriocidal or just bacteriostatic effects. Also, it is extremely difficult, if not impossible, to

accurately compare bacterial inhibition data, even for the same material, between different investigators with this technique because of the host of variables that are involved (Tobias, 1988). Therefore, unless all variables are similar in the study design, each study should be looked at as an entity within itself regarding the data gathered on the materials that were tested. What is needed is the development of an experimental system that more closely approximates the environmental conditions within a preparation and uses dentin as the substrate for the testing. This would provide more clinically relevant data to both investigators and clinicians until the results of clinical testing are released.

### CONCLUSIONS

Within the parameters of this modified cylinder drop plate diffusion assay investigating the antibacterial effects of various dental materials against *S mutans*, *S sobrinus*, *A viscosus*, and *L salivarius*:

1. The primers and adhesives of ProBOND and Syntac and the conditioner and primer of Gluma 3-Step displayed bacterial inhibition against all four bacteria;
2. The resin-modified glass-ionomer cements Fuji Lining LC, Fuji II LC, and Photac-Bond showed bacterial inhibition against all four bacteria; and
3. The polyacid-modified composite resins Infinity and Geristore inhibited only *S mutans* and *L salivarius*, while VariGlass inhibited all four bacteria.

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Table 8. pH Determinations of Primers, Adhesives, and Resins of the Dentin Bond Systems and the Liquid of Fuji Lining LC

Dentin Bond Systems	pH	Composition
<b>Syntac</b>		
Primer	1.2	25% TEG-DMA + 4% maleic acid in acetone and water
Adhesive	3.0	5% PEG-DMA, 5% glutaraldehyde in water
Adhesive without the glutaraldehyde	3.0	
Heliobond resin	4.6	60% BIS-GMA, 40% TEG-DMA
<b>ProBOND</b>		
Primer	2.5	30% HEMA + 6% PENTA in acetone and ethyl alcohol
Adhesive	5.0	5% PENTA, 55% UDMA, TEG-DMA, HEMA, <1% photoinitiators, 0.7% glutaraldehyde
Adhesive without the glutaraldehyde	5.0	
<b>Gluma 3-Step Bonding System</b>		
Conditioner	1.0	1.6% oxalic acid, 2.6% aluminum nitrate, 2.7% glycine water
Primer	3.4	35% HEMA, 5% glutaraldehyde in water
Sealer	5.6	BIS-GMA, photoinitiators
<b>Fuji Lining LC</b>		
Liquid	3.0	HEMA, polyacrylic acid, photoinitiators in water



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**INDIVIDUAL INVESTIGATION (IR) GRANT**

**TITLE: PROTEIN PATTERN RECOGNITION FOR RISK OF PERIODONTAL DISEASE**

**PRINCIPLE INVESTIGATORS: LCDR MICHELE M. D'ALESSANDRO, MSC, USN,  
PH.D. AND GLENN A. MILLER, PH.D.**

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## SECTION A: PROJECT SUMMARY

### ABSTRACT

Our efforts are directed toward the prevention of dental emergencies by predeployment identification of patients at highest risk of periodontal disease. We propose to transition a new, highly discriminatory 2-D polyacrylamide gel electrophoretic (2D-PAGE) method to identify prognostic markers of bacterial and host origin which are associated with the disease. New approaches with better prognostic tools are needed to prioritize patient care and to deliver the most beneficial treatment with reduced resources (ie., periodontists, hygienists, operating dollars).

Gingival crevicular fluid (GCF) and salivary constituents are reported to be specific diagnostic or prognostic markers of oral health. Clinical attachment loss in chronic adult periodontitis has been associated with various indicators of the acute inflammatory response and the cellular immune response in the GCF. Periodontitis appears to progress in a random asynchronous series of bursts of disease activity rather than in a slow continuous manner. Studies to date have concentrated on a restricted number of mediators or components associated with a single episode of disease activity. Identification of markers with specific diagnostic and prognostic capability has not proved successful.

This new 2-D PAGE system allows for a non-invasive method to correlate GCF and salivary proteins with oral health status. Several hundred polypeptide species within clinical samples can be discriminated on a single gel and a polypeptide profile identified for a disease state. Considering the many different proteins which regulate immunity, inflammation, and the function and activity of enzymes that break down gingival tissue, the dental community needs a more comprehensive test to identify the expression or deletion of these biological factors associated with periodontal disease.

The technology is now available for a cost-effective application both in the clinic and in the field. We have the clinical and technical expertise on board to initiate the program. We will, however, require a dedicated research technician to leverage the existing clinical and laboratory assets to accomplish this much needed goal of risk assessment.

We plan to (i) identify and relate 2-D PAGE protein patterns to the status of oral health and disease, and (ii) utilize this technology as the future basis for a non-invasive test of disease activity and prioritization of care.

## KEY PERSONNEL SUMMARY

<u>NAME, RANK/DEGREE</u>	<u>POSITION TITLE or ROLE</u>	<u>DEPARTMENT or ORGANIZATION</u>
D'Alesandro, Michele M. LCDR, MSC, USN, Ph. D	PI	NDS, Research Dept.
Nicoll, Brian K. CDR, DC, USN	PI	NDRI, Det. Bethesda
Miller, Glenn A. Ph.D.	PI	Geo Centers, Inc.

## BUDGET SUMMARY

<u>BUDGET ITEM</u>	<u>1st Yr \$ (FY97)</u>	<u>2nd Yr \$ (FY98)</u>	<u>3rd Yr \$ (FY99)</u>
In-house Personnel	-0-	-0-	-0-
Equipment & Maintenance	-0-	-0-	-0-
Expendable Supplies	15,000	17,690	15,000
Animal Purchase and Per Diem	-0-	-0-	-0-
Travel	2,000	2,000	2,000
Miscellaneous	1,000	2,400	3,850
Contracts/Consultants	-0-	-0-	-0-
<b>TOTAL DIRECT COSTS</b>	<b>\$18,000</b>	<b>\$22,090</b>	<b>\$20,830</b>
<b>TOTAL INDIRECT COSTS</b>	<b>\$45,000</b>	<b>\$49,500</b>	<b>\$55,450</b>
<b>GRAND TOTAL COST</b>	<b>\$63,000</b>	<b>\$71,590</b>	<b>\$76,280</b>

## SECTION B: PROJECT DESCRIPTION

### AIMS AND OBJECTIVES:

The main objective of this proposal is to characterize and correlate the protein patterns in GCF and saliva with the oral health status of the individual. We will use a highly discriminatory two-dimensional polyacrylamide gel electrophoretic (2-D PAGE) system. Specifically we plan to differentiate and diagnose the status of oral health using these 2-D PAGE protein patterns. The specific aims of this proposal are:

#### (1) Patient Identification:

- 1a. Identify the periodontal and endodontic health status of volunteers;
- 1b. Collect GCF and salivary fluid from diseased and healthy individuals.

#### (2) Protein Pattern Analysis:

- 2a. Separate GCF and saliva protein components using 2-D-PAGE;
- 2b. Quantitate the individual protein components of the gel;

- 2c. Compare GCF and salivary protein patterns in healthy and diseased individuals;  
2d. Compare protein patterns and concentrations during disease and after treatment.

## BACKGROUND AND SIGNIFICANCE

Periodontal diseases (periodontitis or periapical periodontitis) can be characterized by gingival inflammation, soft tissue destruction, loss of tooth attachment and loss of bone. They can cause discomfort, malaise, and may also result in systemic infection. Certain individuals and groups, including military personnel, are at high-risk of developing periodontal disease. Navy and Marine Corps operational readiness can be adversely affected due to the extensive treatment required when periodontal disease is diagnosed. (1)

Diagnostic indicators as definitive markers for periodontal disease states have not yet been identified. (2,3) However, the etiology(s) of periodontitis and chronic periradicular lesion formation are generally agreed to be bacterial. (4) The oral bacterial flora is complex. In microbiological terms, a marker for disease is an increase in (i) the number of specific bacteria, (ii) their metabolic activity and (iii) their production of virulence factors. Although the presence of increased numbers of oral species are being evaluated as a diagnostic marker, prognostic reliability has not yet been determined.

Bacteria use several mechanisms to initiate or perpetuate oral disease. In addition, host inflammatory responses appear to play an equally important role in determining disease progression and severity. These two mechanisms provide for a complex interplay between the oral microflora and the state of individual susceptibility. (5) The complexity of the etiology of periodontal disease as a mixed bacterial infection and the evaluation of the host response in the ultimate progression of disease warrants development of more comprehensive tests to identify the expression or deletion of biological factors responsible for disease. This would enable the dental practitioner much greater ability to diagnose and predict oral diseases and to treat them effectively.

More than 50 indicators of the immune and inflammatory response have been identified in GCF. Measurement of the host response in periodontal disease has focused on indicators of (i) the acute inflammatory response (lysosomal enzyme activity, cytoplasmic enzyme activity, and arachidonic acid metabolites), (ii) macrophage activity or cellular immunity (the cytokines), and

(iii) humoral immunity (antibody to putative oral pathogens), all of which have been identified in GCF. Many indicators have been directly related to the presence of periodontal disease (6,7).

Biochemical mediators of inflammation at the site of a periodontal lesion may help to characterize the nature of the immune response. Cytokines (*ie.* IL-1 $\beta$ , IL-2, TNF- $\alpha$ ) in GCF have not been comprehensively evaluated for their relationship to the active phase of periodontal disease. Nevertheless, the presence of these mediators in GCF implicate them in the progression of human periodontal disease.(7)

The heterogeneous nature of the subgingival microflora and the antibody response to these oral microorganisms complicates the role of antibody in the pathogenesis of periodontal disease. Serum and GCF antibody levels are reduced and there are isotype changes in patients with existing periodontal disease. (6,7) The diagnostic significance of specific antibody to suspected periopathogens in GCF is tenuous due to the presence of these same microorganisms at healthy sites. (8) However, identification of specific antibody may prove useful as work progresses using DNA hybridization methodology to identify virulent subspecies.

Components of the complement cascade in oral fluids indicate that C3 conversion is minimal in healthy individuals, and progressively increases in gingivitis, chronic adult periodontitis, rapidly progressive periodontitis, and juvenile periodontitis. (9) C4 cleavage was only observed in GCF from individuals with juvenile periodontitis (LJP). A more comprehensive analysis of complement component cleavage could be achieved using high resolution 2-D PAGE.

The hydrolytic enzyme  $\beta$ -glucuronidase is considered a marker for primary granule release from polymorphonuclear leukocytes (PMN) and is persistently elevated in patients experiencing attachment loss. Elevated numbers of PMN in the crevicular environment are also associated in adult periodontitis with active disease. (7) Multiple molecular forms of other hydrolytic enzymes including gelatinase and type IV collagenase are also present in GCF in adult periodontitis (AP), localized juvenile periodontitis (LJP), and periodontitis associated with diabetes mellitus. Conventional GCF profiles of these proteins, however, do not differentiate between different forms of periodontitis.

Aspartate aminotransferase (AST) activity appears to be site specific and has been positively correlated with the degree of inflammation concurrently with or 3 to 6 months prior to the onset of gingival inflammation. (10) Total alpha-2-macroglobulin (A2M), an acute phase

protein, was elevated in the GCF of patients with active periodontitis. This paralleled an increase in GCF  $\beta$ -glucuronidase activity. In contrast, Skaleric, et al. (11) reported that the concentration of total A2M in GCF was lower in sites with greater inflammation, bone loss, and probing depth.

Host-derived products in GCF are increased in direct response to a bacterial challenge. The combination of bacterial pathogens and host markers may provide a more accurate predictor of disease activity than when either is used independently. There is a close association among specific bacteria, GCF levels of lactate dehydrogenase (LDH), and clinical measures of periodontal disease. (12) LDH levels, probing depth and clinical attachment loss are higher when *P. gingivalis*, *P. intermedia*, *F. nucleatum*, PBGB, and spirochetes are identified at sites of periodontal inflammation. LDH levels may precede detectable clinical change since LDH activity and spirochete numbers returned to baseline levels 3 months after root planing while no detectable clinical change was observed.

Whole saliva contains the secretions from the major and minor salivary glands, microorganisms shed from soft tissue and tooth (plaque) surfaces, and a variety of host cells (epithelial, polymorphonuclear leukocytes) and their products. Special "dip stick" techniques are used to monitor *Streptococcus mutans* and lactobacilli to identify patients at high risk for dental caries. (14)

Host cell products derived from GCF admix with saliva. Examination of whole saliva has a greater diagnostic potential than secretions from individual glands because of the contributions from GCF. (15) Although saliva does not provide the site-specific information that can be derived from GCF, newer more sensitive analytical techniques now permit greater identification of sample constituents.

Few components of saliva have been studied in association with periodontal disease. Total salivary IgA is increased in unstimulated whole saliva in patients with gingivitis and periodontitis and there is a positive relationship with the severity of inflammation. Screening saliva for IgA antibody may prove to be useful in identifying individuals with the potential to develop periodontal disease or those who are currently responding to an infection with *A. actinomycetemcomitans* (16). Parotid IgA antibody levels to *B. gingivalis* were significantly greater in adult periodontitis patients when compared with controls. (17)

Three groups of inhibitors of proteolytic enzymes induced by both supra- and subgingival bacteria, (i) the cystatins (inhibitors of cysteine proteases such as the cathepsins) (18), (ii)



antileucoproteases (inhibitors of both elastase and cathepsin) (19), and (iii) a matrix metalloendoproteinase inhibitor which suppresses the activity of matrix metalloendoproteinases released during the tissue breakdown associated with periodontal inflammation (20), have been identified and characterized in the salivary secretion. These protease inhibitors are potentially important host defense factors and their contribution to periodontal disease warrants further study.

More than 80 enzyme activities have been identified in mixed whole saliva, parotid saliva, serum and neutrophils in people with adult periodontitis, LJP and no disease. In general, the enzyme activity of whole saliva was higher with periodontal disease and it was suggested that bacterial sources accounted for much of this activity. Alkaline phosphatase, esterase, beta-glucuronidase, alpha glucosidase, several aminopeptidases, and trypsin-like activity were present in whole saliva in several patients with periodontal disease. (13) In many instances, there was a significant reduction in esterlipase, aminopeptidases, trypsin and glycosidase activity in whole saliva after periodontal therapy. (21)

#### Summary:

There has been very limited success in the development of a GCF or salivary-based test of oral health status with respect to periodontal disease. Historically, only a small number of diagnostic components have been simultaneously evaluated. Analysis of host mediators in GCF has not demonstrated a consistent correlation between one or a few specific markers and active periodontal disease. The evaluation of multiple factors by using 2 and 3-color single laser flow cytometry has been successful in subcategorizing periodontitis patients into high and moderate-risk groups. (22) Analyzing GCF and saliva with a highly discriminatory 2-D PAGE system to simultaneously monitor and compare their constituents will permit analysis of multiple risk markers to better define oral health status.

#### NAVY / MARINE CORPS RELEVANCE

Identification of those personnel at highest "risk" of developing debilitating dental disease is a major goal of the Naval Dental Research Institute Detachment at the Naval Dental School, Bethesda, MD. The Navy Dental Corps has recently committed itself to a program to increase

operational readiness by prioritizing care and actively seeking out and treating those patients with the highest risk of developing a dental emergency. In order for this approach, the Phase Dentistry Initiative, to be successful, new standards, scientific tools, and methods of risk assessment to manage and ultimately prevent oral disease are needed. Development and testing of new techniques for diagnosis and risk assessment for periodontitis (which is lengthy and costly to treat) is proposed.

The Navy has a limited amount of dental resources and is in the difficult position of not only deciding which patients will receive treatment but also which patients will not. This problem is unique to managed care and of little interest to the civilian community which is interested in delivering maximum dental care to everyone. The ability to identify those individuals which are predisposed to periodontitis will help to (i) minimize treatment because of earlier intervention and the application of preventive strategies for patients at risk, (ii) prioritize treatment for those patients at highest risk and minimize unnecessary, potentially iatrogenic care for others, (iii) conserve and efficiently distribute limited dental resources, and (iv) identify new avenues toward effective periodontal therapy.

Periodontal and endodontic disease indicators in GCF and saliva from gingival sites will be identified. GCF markers will hopefully provide prognostic (ie., risk analysis to periodontal disease susceptibility) and diagnostic (ie., stage of disease progression) information for earlier diagnosis and treatment of these highly destructive diseases.

This new scientific approach to maximize treatment requires a technically feasible yet diagnostically comprehensive indicator of disease status which is not currently available for use by the military clinician. Better evaluation and prioritization of patient treatment is a clear, cost-effective objective to maximize the dental health of military personnel.

## PRELIMINARY RESULTS

The use of 2-D PAGE systems has been used for many years to determine the molecular weight and charge heterogeneity of proteins in complex protein mixtures (e.g. cellular proteins of cells or tissues from different experimental conditions). Only limited use has been made of this procedure for evaluation and characterization of proteins in GCF. (23) Protein pattern changes in animals to assess the toxicological effects of various drugs has been studied using 2-D PAGE (25) These studies, although informative, required a high degree of technical skill and yet did

not always yield the necessary consistency in results. Comparisons of complex patterns has proven to be difficult to assess.

The advantages to sampling GCF include ease of access, atraumatic technique, rapid equilibration with the whole intra-crevice pool, standardization in time and site of sampling and ability for repeat sampling. GCF was collected at selected sites after removal of saliva and supragingival plaque. A durapore strip (pore size = 0.22  $\mu$ m, Millipore Corporation, Bedford, MA) was placed at the entrance of the crevice for 30 seconds. The strip was shaken for 30 seconds in phosphate buffered saline with 3 mM EDTA. Cellular contamination was removed by centrifugation and the supernatants were frozen at 70°C for gel electrophoretic analysis. The protein concentration in the supernatants was determined by the BioRad Protein Assay method. Preliminary studies in our laboratory with non-diseased patients have indicated that it is possible to recover GCF from periopapers in a consistent and quantitative manner. Representative data of GCF protein recovery for two individuals is shown in Table 1. Sufficient amounts of protein are recovered from the periopapers to allow for multiple gel analysis. *These results are important because they assure us that we will be able to collect sufficient sample for replicate runs as we assess changes during the progression and/or treatment of disease.*

Table 1. Protein recovery from gingival crevicular fluid.

Subject	Number of Periostrips	Protein Concentration ( $\mu$ g/ $\mu$ L)	Total Volume ( $\mu$ L)
#1	5	4.00	200
#2	5	3.86	200

*Gel Electrophoresis.* Gel electrophoretic applications include analysis of protein patterns, monitoring purity, and determination of physical characteristics (ie., molecular weight, isoforms, and isoelectric points). The procedure we are using is high resolution 2-D gel electrophoresis which separates proteins by charge in the first dimension (IEF - isoelectric focusing) and by relative mobility of polypeptides in the second dimension (SDS PAGE). Problems with this technique have been related to the inability to obtain reproducible results. This was in part a result of (i) overextension of the first dimension isoelectric focusing gel, and (ii) problems with

maintaining constant and reproducible temperatures within and between runs. The Investigator 2-D Electrophoresis System (Oxford GlycoSystems Inc., Bedford, MA) optimizes temperature, pre-focusing and focusing algorithms, and gel chemistry for consistent results. One of the most significant changes in this procedure is the use of first-dimension gels which contain a fine strand so that the gel may be handled and transferred to the second dimension gel without stretching and deformation of the protein pattern.

High resolution and reproducibility are critical issues in the development of protein data bases. Molecular weight estimates of polypeptides which differ by approximately 350 daltons and isoelectric point estimates which differ by approximately 0.03 pH units can be determined. In addition, over 500 polypeptides per gel have been detected on silver-stained 10% polyacrylamide gels.

Gel electrophoretic markers for molecular weight and charge were prepared and multiple runs were completed to assess the reproducibility of the system. Three molecular weight markers contained several isoforms with respect to their isoelectric point (conalbumin, pI 6.0, 6.3 and 6.6; bovine serum albumin, pI 4.98, 5.07, 5.18; and bovine muscle actin, pI 5.47, 5.50, 5.53). The isoforms were clearly discriminated whether the markers were run alone (Fig 1-A) or with extracted GCF (Fig 1-B).

The presence of several isoelectric points has made the use of these standard 2-D gel markers less than desirable because of the multiple polypeptide spots which could mask the presence of GCF polypeptides. We can successfully run the markers vertically on the left side of the gel for molecular weight determination (data not shown). Although this does not allow for pI markers, markers solely for isoelectric point calibration are now available (BDH, Great Britain) which will allow for vertical analysis of molecular weight in conjunction with independent isoelectric point calibration within the gel.

Silver stained gels are analyzed using a Sun SPARC work station with an XRS Omnimedia Scanner and 2-D Analyzer Software (version 6.1) (Bio Image, Ann Arbor MI). The pI and molecular weight of individual polypeptide spots are determined based on their characteristic migration in comparison to several standards (2-D SDS-PAGE Standards, BioRad Laboratories). The molecular weight range of these proteins is from 17000 to 100000 D. The ampholytes present in the first dimension isoelectric focusing allows for discrimination of pI's between 3.5 and 8.0.

The software package we are using to discriminate polypeptide spots on the gels allows for several ways of presenting the concentration and size of the individual spots for analysis. Figure 2 is a typical surface histogram of a polypeptide cluster routinely observed in GCF from non-diseased individuals. We have the capability to analyze and compare gels or defined sections of gels once they have been scanned and added to the database. The spots integrated intensity, area, pI, and molecular weight are all part of the spot database which is generated. The relative concentration of individual polypeptides within a gel or among a series of gels can then be determined by a query of the intensity ratios based on quantitative ratios of matched spots. *Therefore, if changes are readily observed in a specified area of the gel, we also have the capability to limit our analysis based on the molecular weight and isoelectric point markers which define the area.*

In conclusion, we have (i) confirmed that this methodology is highly reproducible between runs with respect to standard markers of molecular weight and isoelectric point (Fig 1-A, 1-B); (ii) confirmed that it is possible to discriminate individual differences in the number and relative concentration of polypeptides in GCF extracts (Fig. 2); (iii) determined optimal conditions for separation with respect to water purity and product specifications and identification of GCF protein constituents. *Collectively, these accomplishments position us well to undertake the studies that we now propose.*

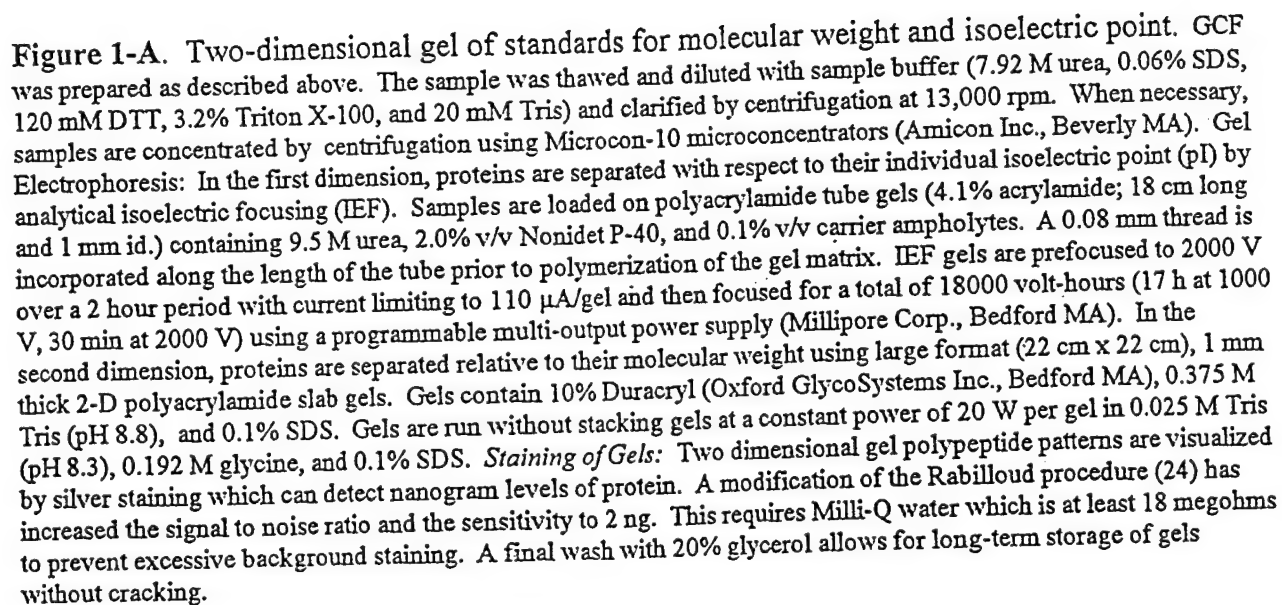


Figure 1-A. Two-dimensional gel of standards for molecular weight and isoelectric point. GCF was prepared as described above. The sample was thawed and diluted with sample buffer (7.92 M urea, 0.06% SDS, 120 mM DTT, 3.2% Triton X-100, and 20 mM Tris) and clarified by centrifugation at 13,000 rpm. When necessary, samples are concentrated by centrifugation using Microcon-10 microconcentrators (Amicon Inc., Beverly MA). Gel Electrophoresis: In the first dimension, proteins are separated with respect to their individual isoelectric point (pI) by analytical isoelectric focusing (IEF). Samples are loaded on polyacrylamide tube gels (4.1% acrylamide; 18 cm long and 1 mm id.) containing 9.5 M urea, 2.0% v/v Nonidet P-40, and 0.1% v/v carrier ampholytes. A 0.08 mm thread is incorporated along the length of the tube prior to polymerization of the gel matrix. IEF gels are prefocused to 2000 V over a 2 hour period with current limiting to 110  $\mu$ A/gel and then focused for a total of 18000 volt-hours (17 h at 1000 V, 30 min at 2000 V) using a programmable multi-output power supply (Millipore Corp., Bedford MA). In the second dimension, proteins are separated relative to their molecular weight using large format (22 cm x 22 cm), 1 mm thick 2-D polyacrylamide slab gels. Gels contain 10% Duracryl (Oxford GlycoSystems Inc., Bedford MA), 0.375 M Tris (pH 8.8), and 0.1% SDS. Gels are run without stacking gels at a constant power of 20 W per gel in 0.025 M Tris (pH 8.3), 0.192 M glycine, and 0.1% SDS. *Staining of Gels:* Two dimensional gel polypeptide patterns are visualized by silver staining which can detect nanogram levels of protein. A modification of the Rabilloud procedure (24) has increased the signal to noise ratio and the sensitivity to 2 ng. This requires Milli-Q water which is at least 18 megohms to prevent excessive background staining. A final wash with 20% glycerol allows for long-term storage of gels without cracking.

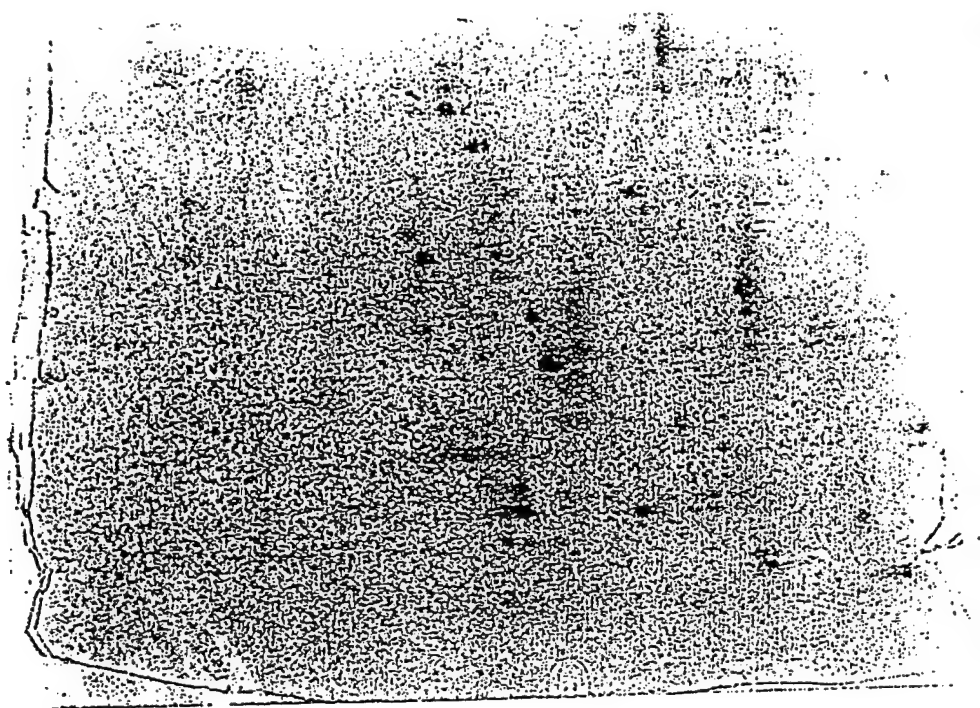


Figure 1-B. Two-dimensional gel of GCF from a non-diseased individual. Preparation of the gel, electrophoresis, and staining of the gel are as described in Figure 1-A.

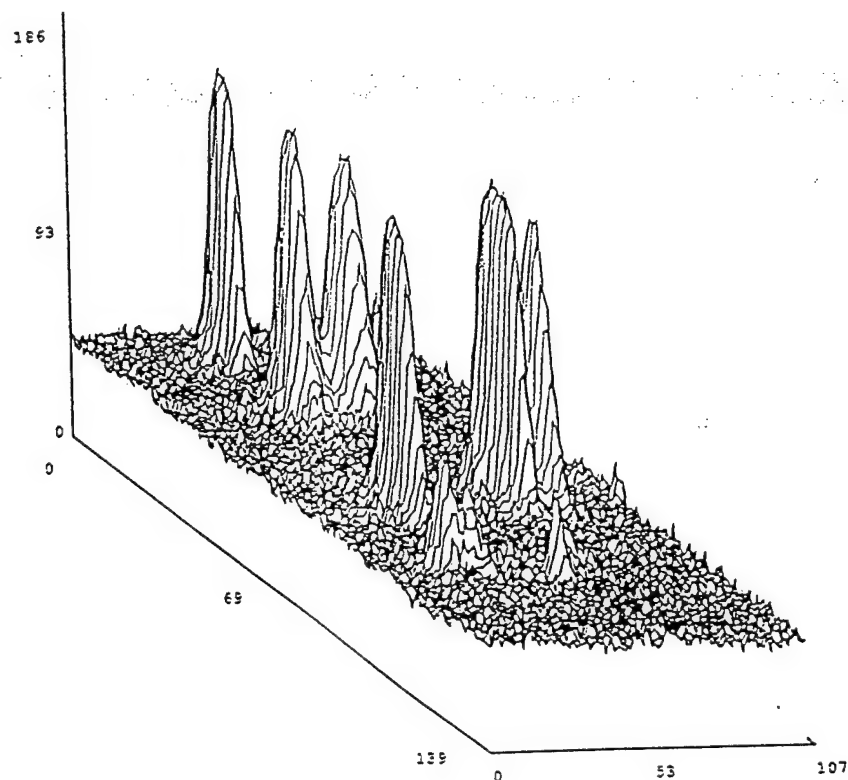


Figure 2. Surface histogram of a typical polypeptide cluster defined in the upper left quadrant of a large format (22 cm  $\times$  22 cm) 10% Duracryl (Oxford GlycoSystems Inc., Bedford MA) 2-D gel.



## EXPERIMENTAL DESIGN AND METHODS

### Approach to Specific Aim 1. (a): Identification of oral health status of volunteers.

All subjects will undergo a comprehensive dental examination by a calibrated periodontist for full mouth site by site analysis of the periodontal condition which will include contributory factors. Data will be collected using the *Periodontal Site Characterization Data Collection Set* (Appendix A).

### Approach to Specific Aim 1. (b): Collecting salivary and gingival crevicular fluids from diseased and healthy individuals

#### Control Population:

GCF and saliva samples will be obtained from 12 subjects with clinically healthy periodontium for use as controls. These subjects will have minimal loss of attachment as determined by the same comprehensive periodontal examination for disease patients. (Appendix A: *Periodontal Site Characterization Data Collection Set*)

#### Periodontitis patients:

Subjects with various degrees of periodontal disease will be selected from both male and female patients referred to the Periodontics Clinic at the National Naval Dental Center. The major criterion for selection will be evidence of severe loss of periodontal attachment at any two or more teeth where, in the opinion of the attending periodontist, the loss of attachment is due to a recent or current episode of juvenile, adult, or rapidly progressing periodontitis. Other forms of periodontal diseases will be excluded from the study. Subjects should be in an active stage of the disease cycle at some sites. A full medical history must be available for each subject. The following conditions will specifically exclude individuals from the study:

- Pregnancy
- Antibiotic or long-term anti-inflammatory therapy in the previous three months
- Clinically significant systemic diseases that may affect healing or preclude surgical treatment (i.e., uncontrolled diabetics)
- Systemic administration of anti-neoplastic, immunostimulating, or immunosuppressive agents
- Refusal or inability to sign informed consent
- Participation in another clinical trial using an investigational new drug or device within 30 days of entrance into this study.
- Conditions where venipuncture is contra-indicated.

Sampling routine: Clinical examinations for site characterization, GCF, and saliva sampling (primarily for periodontitis patients) will be performed:

- a. On entry to the study (prior to commencement of therapy).
- b. On completion of hygiene therapy.
- c. Twelve weeks after completion of all surgery or after completion of hygiene therapy if surgery is not performed.
- d. Six months after completion of all treatment, at recall.



Patients will have GCF and saliva samples taken by a dentist four times during the course of the study. GCF sampling is described in the Preliminary Results section. Saliva will be sampled and processed as previously described (18).

**Approach to Specific Aim 2. (a):** *Separating GCF and saliva samples into their respective protein components using sophisticated 2-D-PAGE.*

We will use a highly discriminating 2-D-PAGE methodology available with the Investigator 2-D Electrophoresis System (Oxford GlycoSystems Inc., Bedford, MA). There will be simultaneous identification and quantitation of a large number of protein components. Patterns of healthy and diseased states will then be compared. See Preliminary Results section.

**Approach to Specific Aim 2. (b):** *Quantitating individual protein components of the gel.*

Gel patterns will be analyzed using a Sun SPARC work station. This system permits identification and quantification of spots, assignment of isoelectric points and molecular weights, and comparison of different gel patterns for matching protein spots. Images from the database can be compared to effectively track changes in protein content between patients and/or during disease progression.

**Approach to Specific Aim 2. (c):** *Comparing protein patterns obtained from healthy and diseased individuals to evaluate specific markers of periodontal disease.*

Once protein scatter patterns are identified and indexed, patterns obtained using samples from normal individuals will be compared with those from diseased individuals by computer subtraction using 2-D Analyzer Software version 6.1 (Bio Image, Ann Arbor MI). See Specific Aim 2. (b). Proteins common to both the normal and diseased state, as well as proteins unique to either state can be identified.

**Approach to Specific Aim 2. (d):** *Comparison of changes in patterns during progression of disease or after therapy.*

Comparison of changes in patterns and in concentrations will be done as indicated in the approach to Specific Aim 2. (c).

## **RESEARCH TRANSITION PLAN**

As new technical abilities in the early identification of personnel at risk of developing periodontal disease are realized, the dental community will be better equipped to support the fleet and enhance operational readiness through decreased loss of manpower and manhours due to complications associated with periodontal disease. Once normal and diseased protein patterns are determined and specific markers of predisposition and/or disease progression are determined, the technology can be considered a diagnostic technique when considering the logistics of immediate and future treatment regimens.

The scientific level and skill required to achieve maximum efficient use of this technology would be similar to that currently in use in our Navy Drug Screening labs. The technology would be best utilized within a laboratory where it is the focus of the labs mission. This is feasible since the fluids that would be analyzed (ie., GCF and saliva) are easily obtained (even if at remote sites) and can be frozen and shipped for analysis.

**PATENT DISCLOSURES AND INVENTIONS:** None anticipated.

**MILESTONES: Protein components in oral secretions and the status of oral health.**

1st Yr FY97				2nd Yr FY98				3rd Yr FY99			
1	2	3	4	1	2	3	4	1	2	3	4

**1. Identification of protein constituents in oral secretions.**

1. (a) Identify a control population  
S-----C
1. (b) Separate saliva and GCF proteins on 2-D PAGE  
S-----C--R
1. (c) Quantitate individual protein components  
S-----C-----R

**2. Relationship of various protein patterns in healthy and diseased patients.**

2. (a) Identify health status of patients  
S-----C
2. (b) Collect GCF and saliva from patients  
S-----C
2. (c) Separate saliva and GCF proteins on 2-D PAGE  
S-----C--R
2. (d) Compare protein patterns in patients  
S-----C--R
2. (e) Compare changes in protein patterns in healthy and diseased patients  
S-----C--R

**Legend:** Planned milestones    S = Start    C = Complete    T = Terminate    R = Report

## SECTION C: LITERATURE SEARCH

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DTIC: A DTIC search was performed under DTIC request numbers GIO32D (Technical Reports) and GIO32F (Workunits) on 06 December 1994. The proposed work will not result in any undue duplication of technical effort previously performed by other organizations. There were zero (0) finds.

#### KEYWORDS:

*Scientific/Technical:* gingivitis, periodontitis, gingival crevicular fluid, saliva, cytokines, periradicular lesions, inflammation, humoral immune response, cellular immune response,  $\beta$ -glucuronidase.

*Methodological:* two dimensional polyacrylamide gel electrophoresis, isoelectric focusing, image analysis, periodontal disease, periapical surgery, pocket depth, silver staining.

## SECTION D: BIOGRAPHICAL SKETCH

**LCDR D'Alesandro, MSC, USN**, is a Research Biochemist with over 25 publications in peer-reviewed journals. Since completion of her graduate studies at SUNY Buffalo (Department of Experimental Therapeutics, Roswell Park Cancer Institute, Biochemistry Division), she has held positions at the Armed Forces Radiobiology Research Institute (Experimental Hematology, Research Biochemist), the Naval Medical Research Institute (Thermal Stress Division, Biochemistry Officer, IR Work Unit 61152NMR0001.0011378 Oct 1989 - September 1990), and the United States Naval Academy (Chemistry Department, Associate Chairman). Her most current research endeavors have culminated in 13 publications dealing with hormone-associated cold weather acclimation in operational forces with COL H. Lester Reed, MC, USA (Chief, Dept. Medicine, Madigan Army Medical Center). At AFRRI, she was a co-investigator on both the canine septic shock and rat combined injury (burn, radiation) program.

**CDR Brian K. Nicoll, DC, USN** (Diplomate, American Board of Periodontology) is currently OIC of the NDRI and assistant professor at the Naval Dental School. Previous assignments include positions as Clinic Director and staff periodontist 3rd Dental Battalion, Okinawa; staff periodontist, NDC San Francisco, Chief, Oral Diagnosis, USNCD Subic Bay, RP; Clinic Director, NDC, Great Lakes; and Division Officer, USS KITTY HAWK (CV-63). Postgraduate residency training was completed at NH Oakland (general dentistry) and at NDS Bethesda (periodontics). Adjunct clinical association are held with University of the Pacific, University of California, San Francisco, and George Washington University School of Medicine.

**Dr. Glenn A. Miller** is an Immunologist/Microbiologist with over 30 publications in peer-reviewed journals. He has an extensive background in microbiology, immunology, and molecular biology in both industry and academia. He has also managed programs in monoclonal antibody and vaccine development. He is currently Professor at the Naval Dental School and hold adjunct faculty positions at the Johns Hopkins University, George Washington University and The Medical College of Virginia. As a senior research scientist with GEO-CENTERS, INC., Dr. Miller is responsible for initiating and developing microbiologically and immunobiologically oriented research directed at understanding problems of dental and oral health in the Research Department of the National Naval Dental Center, Bethesda, MD. He is currently the principle investigator for studies relating to the role of polyclonal B-cell activators and superantigens in the pathogenesis of periodontal disease. In addition, he also directs work toward determining the involvement of interleukin-1 and tumor necrosis factor in the development of periodontal disease.

## SECTION E: OTHER SUPPORT

None

## SECTION F: ENVIRONMENT

**FACILITIES:** No special facilities or alterations to existing facilities required.

**PERSONNEL AND ENVIRONMENTAL HAZARDS/PRECAUTIONS:** No procedures, materials, chemicals, biologics, or situations that produce personnel or environmental hazards beyond potential routine laboratory exposures are anticipated.

## SECTION G: RESOURCES REQUIRED

### DETAILED BUDGET FOR THE FIRST YEAR

DIRECT COSTS		
<i>IN-HOUSE PERSONNEL:</i>		
<u>Rank/Name/Grade or Degree</u>	<u>Effort</u>	<u>Accelerated</u>
	<u>(%/Yr)</u>	<u>Salary/Yr</u>
		<u>(\$)</u>
<u>Professional</u>		
LCDR Michele M. D'Alesandro, MSC Ph D.	40%	0
CDR Brian Nicoll, DC	10%	0
<u>Support</u>		
		0
YEAR 1 TOTAL IN-HOUSE PERSONNEL COSTS		0
<i>EQUIPMENT AND MAINTENANCE (\$):</i>		
YEAR 1 TOTAL EQUIPMENT COSTS		0
<i>EXPENDABLE SUPPLIES (\$):</i>		
2-D Gel Electrophoresis Supplies		\$13,500
Protein Assay Kits		\$ 500
Silver Staining Kits		\$ 1,000
YEAR 1 TOTAL EXPENDABLE SUPPLIES COSTS		\$15,000
<i>ANIMAL PURCHASE AND PER DIEM (\$):</i>		
		0
<i>TRAVEL (\$):</i>		
American Association of Dental Research		\$ 1,000
Federation of American Societies of Experimental Biologists		\$ 1,000
YEAR 1 TOTAL TRAVEL COSTS		\$ 2,000
<i>MISCELLANEOUS (\$):</i>		
Computer program updates		\$ 1,000
YEAR 1 TOTAL MISCELLANEOUS COSTS		\$ 1,000
<i>CONTRACTS / CONSULTANTS (\$):</i>		
		0
YEAR ONE TOTAL DIRECT COSTS		\$18,000

## INDIRECT COSTS

### IN-HOUSE PERSONNEL (\$):

<u>Item</u>	<u>Rate</u>	<u>Cost</u>
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YEAR 1 MILITARY / CIVIL SERVICE INDIRECT COSTS		0
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### IN-HOUSE CONTRACTOR PERSONNEL:

<u>Name/Degree</u>	<u>Organization</u>	<u>Effort</u> (% Yr)	<u>Distribute Overhead</u> (Y/N)
Glenn Miller, Ph D.	Geo Centers Inc.	20%	N
New Hire	Geo Centers Inc	100%	Y

### IN-HOUSE CONTRACTOR PERSONNEL (\$):

<u>Item</u>	<u>Rate</u>	<u>Cost</u>
Research Technician		\$45,000

YEAR 1 CONTRACTOR PERSONNEL INDIRECT COSTS	\$45,000
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YEAR 1 TOTAL DIRECT COSTS

\$18,000

YEAR 1 TOTAL INDIRECT COSTS

\$45,000

YEAR 1 TOTAL COSTS

\$63,000

A 20% increase would allow us to perform more sophisticated enzymatic assays using monoclonal antibodies once protein patterns are identified. A 20% decrease would prevent us from hiring the technical expertise required to complete the project.

### PERSONNEL AND ORGANIZATION

LCDR Michele M. D'Alesandro (Research Biochemist) will be specifically responsible for the technical implementation of the 2-D PAGE system and protein pattern analysis of oral fluids [Specific Aim 2. (a) (b) (c) (d)].

CDR Brian K. Nicoll (Diplomate, American Board of Periodontology) will be specifically responsible for verifying the dental health status of volunteers and collection of oral fluids [Specific Aim 1. (a) (b)].

Dr. Glenn A. Miller (Microbiologist, Immunologist) will be specifically involved with pattern analysis of the proteins separated by 2-D PAGE [Specific Aim 2. (a) (b) (c) (d)].

CONTRACTS, CONSULTANTS, COLLABORATORS, CONSORTIA: None



## Appendix A: *Periodontal Site Characterization Data Collection Set*

A full examination will be performed to include:

- Teeth present
- Site specific restorative and caries status (lesions, faulty restorations)
- Recent (within 12 months) bitewing radiographs

### *Periodontal Status:*

- Supragingival plaque
- Supragingival calculus
- Probing pocket depth
- Probing attachment level
- Gingival recession/enlargement
- Bleeding/suppuratation on probing
- Subgingival calculus

The above periodontal measurements will be made on all erupted teeth (except third molars), at four sites per tooth (mesiobuccal, mesiolingual, distobuccal, and distolingual) except for teeth #'s 2, 3, 14, 15, 18, 19, 30, and 31 at which additional measurements will be made at the mid-buccal and mid-lingual sites.

The following measurements will be taken in the order indicated:

### Supragingival Plaque

- 0 - Absent after both visual and gentle probing of tooth surface
- 1 - Present on probing
- 2 - Visibly present
- 3 - Abundant visible deposits
- 9 - Cannot be scored/tooth missing

### Supragingival Calculus

- 0 - Absent
- 1 - Present
- 9 - Tooth missing/cannot be scored

Probing Pocket Depth Probing pocket depth is defined as the distance from gingival margin to the base of the periodontal pocket and will be measured with a ball-ended probe of WHO 621 dimensions, modified by Williams markings, at a force of .25N. Pocket depth measurement will be made to the nearest millimeter with

the probe directed along the longitudinal axis of the tooth, placed to the deepest penetration into the periodontal pocket. In cases where doubt exists such as a marked divergence from the parallel between the probe graduations and the gingival margin the lowest score is to be recorded. *(The type of pocket depth probe may need to be modified in the light of the training and calibration exercises)*

Probing attachment level Clinical attachment level is defined as the distance from the tooth cemento-enamel junction (CEJ) or "neck of the tooth" to the base of the periodontal pocket (the deepest level of gum attachment loss on the tooth). Attachment level and probing depth will be measured at the same sites. This dimension will be recorded either directly, as in the presence of gingival recession, or indirectly in cases of no recession or the presence of gingival edema or hyperplasia. Direct measurements will be made by measuring the probing distance between the observed CEJ and the base of the periodontal pocket. Indirect measurements will be determined by 1) in the case of gingival enlargement, subtracting the measurement of the distance from the gingival margin to the CEJ detected by the probe tip (tactile) or through visualization from measured pocket depth or 2) in the case of gingival recession, adding the distance from the gingival margin to the visible CEJ to the measured pocket depth. The tactile sensation of a ball-ended probe (WHO 621 or CPITN-C) should be used. *The presence of subgingival calculus deposits may introduce error into probing measurements of either attachment level or probing depth.*

Bleeding on probing On completion of probing in each quadrant bleeding is observed as follows:

- 0 - Nil
- 1 - Pinpoint with delay
- 2 - Rapid
- 3 - Spontaneous *without probing*
- 9 - cannot be scored/tooth missing

Suppuration on probing On completion of probing in each quadrant bleeding is observed as follows:

- 0 - Nil
- 1 - Pinpoint with delay
- 2 - Rapid
- 3 - Spontaneous *without probing*
- 9 - cannot be scored/tooth missing

Subgingival Calculus. Determined with a ball-ended probe (WHO 621)

- 0 - Absent
- 1 - Present

## CURRICULUM VITAE

**MICHELE M. D'ALESSANDRO, LCDR, MSC, USN**

**Position**                      **Division Officer**  
**Research Department**  
**Naval Dental School**  
**Bethesda, Maryland 20889-5077**

### Academic Training

1976              B.S., Biology, S.U.N.Y. Fredonia  
1978              M.S., Biology, S.U.N.Y. Fredonia  
1983              Ph.D., Biochemistry, S.U.N.Y. Buffalo.

### Military Training

1995              Department Head Course, Naval School Health Sciences, Bethesda, MD.  
1995              Alcohol and Drug Abuse Management Training, Naval District Washington  
1990              Intermediate Leadership and Management Education and Training Course, Naval School Health Sciences, Bethesda, MD.  
1984              Medical Effects of Nuclear Weapons Course, Armed Forces Radiobiology Research Institute, Bethesda, MD.  
1983              Officer Indoctrination School, NETC, Newport, RI.

### Awards

1985              Joint Meritorious Unit Award  
1990              Antarctic Service Medal  
1991              National Defense Service Ribbon  
1992              Navy Unit Commendation  
1994              Navy Commendation Medal

### Professional Experience

1976-78              Research/Teaching Assistant, Department of Biology, State University of New York at Fredonia.  
1978-83              Research Affiliate/Predoctoral Candidate, Roswell Park Cancer Institute, Grace Cancer Drug Center, State University of New York at Buffalo.  
1981-83              Assistant Professor, Science Department, Erie Community College, Orchard Park, NY.

- 1983-87                      Research Biochemist, Armed Forces Radiobiology Research Institute,  
Experimental Hematology Department, Bethesda, MD.
- 1987-1991                    Biochemistry Officer, Naval Medical Research Institute, Thermal Stress  
Program, Bethesda, MD.
- 1991-1994                    Associate Chairman/Associate Professor, Chemistry Department,  
United States Naval Academy, Annapolis, MD.
- 1994-present                Division Officer, Research Department, Naval Dental Research Institute  
Detachment Bethesda  
Associate Professor, Naval Dental School, Bethesda, MD.

### Professional Affiliations

Sigma Xi - Society of American Scientists  
Society of Armed Forces Medical Laboratory Scientists  
American Society for Circumpolar Health

### Publications

D'Alesandro MM, Jaskot RH, Dunham VL. Soluble and chromatin bound DNA polymerases in developing soybean. Biochemical Biophysical Research Communications 1980;94:233-239.

D'Alesandro MM, Srivastava BIS. Poly(a) polymerase and (ADP-ribose) polymerase activities in normal and crown gall tumor cultures of tobacco. FEBS Lett 1985;188:239-242.

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Gruber DF, D'Alesandro MM. Alteration of rat polymorphonuclear leukocyte function following thermal injury. Journal of Burn Care & Rehabilitation 1989;10:394-401.

Lewis SB, Thomas JR, Schrot J, Ahlers ST, Armstrong DW, Van Orden KF, Reed HL, Hetslink RL, D'Alesandro MM, Homer LD. Cold: An operational hazard. US Navy Classified Symposium on Arctic/Cold Weather Operational Support for Surface Ships, Silver Spring, MD. 29-30 November 1989.

D'Alesandro MM, Gruber DF, Reed HL, O'Halloran KP, Robertson R. Effects of collection methods and storage on the in vitro stability of canine plasma catecholamines. American Journal of Veterinary Research 1990;51:257-259.

D'Alesandro MM, Reed HL, Robertson R, Lewis SB. Simplified methods of collecting and processing whole blood for quantitation of plasma catecholamines. Laboratory Medicine 1990;21:26-29.

D'Alesandro MM, Gruber DF. Quantitative and functional alterations of peripheral blood neutrophils after 10% and 30% thermal injury. Journal of Burn Care & Rehabilitation 1990;11:295-300.

Gruber DF, O'Halloran KP, D'Alesandro MM, Farese AM. Hypermetabolic priming of canine neutrophils by 7-S nerve growth factor. American Journal of Veterinary Research 1990;51:921-923.

Reed HL, Brice D, Shakir KMM, Burman KD, D'Alesandro MM, O'Brian JT. Decreased free fraction of thyroid hormones after prolonged Antarctic residence. Journal of Applied Physiology 1990;69:1467-1472.

Reed HL, D'Alesandro MM. Changes in thyroid hormone status with antarctic residence. Antarctic Journal of the United States 1990 Review Issue.

D'Alesandro MM. (Contributing Author) "Principles for the Conduct of Research in the Arctic". Prepared by the Social Science Task Force of the U.S. Interagency Arctic Research Policy Committee. Arctic Research of the United States. 1990;4:105-106.

D'Alesandro MM, Gruber DF, O'Halloran KP, MacVittie TJ. In vitro modulation of canine polymorphonuclear leukocyte function by GM-CSF. Biotherapy 1991;3:233-239.

Reed HL, Kowalski KR, D'Alesandro MM, Robertson R, Lewis SB. Propranolol fails to lower the increased blood pressure caused by cold air exposure. Aviation, Space and Environmental Medicine 1991;62:111-115.

D'Alesandro MM, Malik MJ, Reed HL. Plasma catecholamine degradation with longterm storage. Naval Medical Research Institute 91-01, 1991.

Reed HL, D'Alesandro MM, Harford R. Polar T3 Syndrome: Meaning for midlatitude residents. Antarctic Journal of the United States 1991;26(5):239-240.

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Hesslink RL, D'Alesandro MM, Armstrong DW, Reed HL. Human cold air habituation is independent of thyroxine and thyrotropin. J Appl Physiol 1992;72(6):2134-2139.

Harford RR, Reed HL, Morris MT, Sapien IE, Warden R, D'Alesandro MM. Relationship between changes in serum thyrotropin and total and lipoprotein cholesterol with prolonged Antarctic residence. Metabolism 1993;42:1159-1163.

Reed HL, Quesada M, Hesslink RL, D'Alesandro MM, Christopherson RJ, Turner BV, Young BA. Changes in serum triiodothyronine (T3) kinetics and hepatic type I 5'-deiodinase activity of cold exposed swine. Amer J Physiol (Endocrinol and Metabolism) 1994;266:E786-E795.

D'Alesandro MM, Reed HL, Malik M, Homer LD. Changes in triiodothyronine (T3) mononuclear leukocyte receptor kinetics after T3 administration and multiple cold air exposures. Receptor 1994;4:259-268.

Miller GA, Hickey MF, D'Alesandro MM, Nicoll BK. Functional studies on long-term cryopreserved peripheral blood mononuclear cells. Clin Diagnostic Lab Immun (Submitted for publication).

Miller GA, Canaan TJ, D'Alesandro MM. Functional immune responses of cryopreserved human lymphocytes. (Manuscript in preparation).

D'Alesandro MM, Reed HL, Malik M, Quesada M, Hesslink R, Homer LD, Young B. Characteristics of the porcine mononuclear leukocyte nuclear triiodothyronine receptor and the effect of cold exposure on receptor kinetics. Amer J Vet Research (Manuscript in preparation).

#### Abstracts

D'Alesandro MM, Jaskot RH, Dunham VL. DNA polymerase A in developing soybean embryo. Plant Physiology 1978;61:61.

D'Alesandro MM, Gruber DF. Murine model of radiation or thermally induced non-specific immuno-dysfunction. Journal Leukocyte Biology 1986;40:325.

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Patchen ML, D'Alesandro MM, Brook I. Cellular mechanisms involved in glucan-mediated survival in irradiated mice: mechanisms in addition to hematopoietic stem cell regeneration. Experimental Hematology 1986;14:541.

Patchen ML, MacVittie TJ, D'Alesandro MM. Radiation induced hematologic, nonspecific immunological and humoral effects in the canine. 4th International Symposium on Bioluminescence and Chemiluminescence, Friedberg, Germany. 1986.

MacVittie TJ, D'Alesandro MM, Monroy RL, Farese A, Patchen ML, Clark SC, Donahue RE. Stimulation of hematopoiesis in canine by in vitro administration of recombinant human GM-CSF (rhGM-CSF). J Cellular Biochem Suppl 1988;12A:152a.

D'Alesandro MM, Reed HL, Robertson R, Lewis SB. Simplified methods of collecting and processing whole blood for quantitation of plasma catecholamines. FASEB March 1989

Reed HL, Lewis SB, Kowalski KR, D'Alesandro MM, Robertson R, Homer L. Propranolol administration accentuates the pressor response to cold air challenge in normal men. FASEB March 1989.

D'Alesandro MM, Reed HL, Malik MJ, Homer L. Nuclear thyroid hormone receptors: Changes in receptor kinetics in mononuclear leukocytes after multiple cold air exposure. FASEB April 1990.

Hesslink R, D'Alesandro M, Castro S, Armstrong D, Kowalski K, Reed H. Is thyroxine ( $T_4$ ) important for human cold acclimation? FASEB April 1990.

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Collins DL, Cohen SJ, Gruber DF, D'Alesandro MM, Mickley DA. Isolation suppresses both immune and neuroendocrine functioning and decreases the locomotor activity of normal and immunosuppressed C3h/HeN mice. Society for Neuroscience, 1990;16:390.

Lopez A, Reed L, D'Alesandro M. Changes in hematological profiles during winter field operations. FASEB April 1991.

D'Alesandro M, Reed L, Malik M, Quesada M, Hesslink R, Castro L, Homer L, Young B. Porcine mononuclear leukocyte nuclear thyroid hormone receptors: effects of cold exposure on receptor kinetics. FASEB April 1991.



Reed L, Quesada M, Cosgrove S, D'Alesandro M, Harford R, Castro S, Turner B, Christopherson R, Young B. Changes in porcine serum triiodothyronine (T<sub>3</sub>) kinetics with prolonged exposure to cold. FASEB April 1992.

Harford R, Reed L, Morris M, Sapien I, Warden R, Kowalski K, Lopez A, D'Alesandro M. Alteration in thyrotropin, total and lipoprotein cholesterol with Antarctic residence. FASEB April 1992.

Quesada MH, Reed HL, Cosgrove S, Licaucou G, Castro S, D'Alesandro M, Homer L, Young B. Changes in porcine triiodothyronine distribution after prolonged cold exposure versus a short-term exposure. FASEB April 1992.

Malik MM, D'Alesandro MM, Reed HL, Lopez A, Harford R, Homer L. Mononuclear leukocyte nuclear thyroid hormone receptors: Effects of prolonged Antarctic residence on receptor kinetics. FASEB April 1995.

Miller GA, D'Alesandro MM, Xinbin Gu, Diehl S. Evidence for superantigen production by bacteria associated with adult periodontitis. 9th International Congress of Immunology, July 1995.

Xinbin G, Miller GA, D'Alesandro MM, Diehl SR. T-cell receptor V $\beta$  gene expression after stimulation of human lymphocytes with components of periodontal pathogens. NIH Research Festival, September 1995.

Miller GA, Walsh R, D'Alesandro MM. Influence of *Treponema denticola* on cytokine production by peripheral blood lymphocytes from patients with advanced periodontitis. J Dent Res 75 (IADR Abstracts):642, 1996.

Miller GA, D'Alesandro MM, Euler G. Production of interleukin-6 by polymorphonuclear leukocytes in gingival and periradicular tissues. FASEB, AAI June 1996.

#### Presentations:

D'Alesandro MM, Patchen M, Solberg B, MacVittie TJ. Effects of particulate glucan on murine peritoneal macrophage 5'-nucleotidase, leucine aminopeptidase, and alkaline phosphodiesterase ectoenzyme activity. 3rd International Conference on Immunopharmacology Florence, Italy, 1985.

D'Alesandro MM, Gruber DF, Patchen ML, MacVittie TJ. Oxidative response capabilities of neutrophils from a canine model of gram negative sepsis. International Conference on Analytical Cytology XI. Abstract No. 169. 1985.

D'Alesandro MM, MacVittie TJ, Kaffenberger W, Fink MP, Bowles C, Natanson C, Patchen ML, Parrillo J, Sauber J, Conklin JJ, Walker RI. Canine hyperdynamic sepsis: Neutrophil membrane potential, metabolic and functional defects. Immune Consequences of Thermal and Traumatic Injury. Snowbird, Utah, 1987.



MacVittie TJ, D'Alesandro MM, Fink MP, Patchen ML, Natanson C, Bowles C, Sauber J, Parrillo J, Conklin JJ, Walker RI. Hyperdynamic gram negative sepsis in the canine: Hematological and hemopoietic effects. Immune Consequences of Thermal and Traumatic Injury. Snowbird, Utah, 1987.

Reed HL, Kowalski KR, D'Alesandro MM, Robertson R, Lewis SB, Homer L. Propranolol accentuates the pressor response to acute cold air challenge in normal men. First Navy Independent Exploratory Development Symposium, CPIA 492, June 1988, Volume 1.

D'Alesandro MM, Reed HL, Malik MJ, Homer L. Nuclear thyroid hormone receptors: Changes in receptor kinetics in mononuclear leukocytes after multiple cold air exposure. 8th International Congress on Circumpolar Health, Whitehorse, Yukon, Canada. May 20-25, 1990.

D'Alesandro M, Reed HL, Hesslink RL, Harford R. Cold exposure and human adaptation. Third Navy Independent Research/Independent Exploratory Development Symposium, CPIA, June 1990.

### Related Training

Immunoperoxidase Workshop, DAKO Corporation, 1984.

Separation Techniques: Proteins and other Biomolecules, Center for Advanced Training in Cell and Molecular Biology, Catholic University, Washington, DC. 1985.

Methods Course in Clinical Flow Cytometry, Smith, Kline and French and the Hershey Medical Center. 1985.

Advances in Morphometry and Polidy Determination. Armed Forces Institute of Pathology, Washington, DC. 1985.

Receptor Binding Techniques, Center for Advanced Training in Cell and Molecular Biology, Catholic University, Washington, DC. 1985.

Study of Differentiation in Culture, Center for Advanced Training in Cell and Molecular Biology, Catholic University, Washington, DC. 1986.

Biological Applications for High Pressure Liquid Chromatography, Waters Millipore. 1987.

Laser Safety: Hazard, Inspection, and Control. Laser Institute of America, Cincinnati, OH. 1988.

2-D Electrophoretic Analysis, Bio Image, Ann Arbor MI. 1995

### Project Funding

National Science Foundation, Department of Polar Programs, Number 8817037, Grant S-030.  
"The Influence of Prolonged Polar Residence Upon the Cellular Distribution of Thyroid  
Hormones", July 1989 - July 1991.

Naval Medical Research and Development Command, Independent Research Work Unit  
61152NMR00001.0011378. "Effects of chronic cold exposure on the cellular distribution of  
human thyroid hormones", October 1989 - September 1990.

Naval Medical Research and Development Command, Work Unit 6233NN MM33C30.004-  
1002. "The role of pharmacological intervention in improving responses to cold challenge",  
October 1989 - June 1991.

20 Feb 1996

**CURRICULUM VITAE**  
revised January 1996

***PERSONAL INFORMATION***

Brian Keith Nicoll, Commander, Dental Corps, United States Navy

Current Business Address: Research Department  
Naval Dental School  
National Naval Dental Center  
8901 Wisconsin Avenue  
Bethesda, Maryland 20889-5602

Current Home Address: 19115 Wheatfield Drive  
Germantown, Maryland 20876

Business Telephone: (301) 295-0180  
Home Telephone: (301) 972-0456

Date and Place of Birth: 26 September 1954  
San Francisco, California

Marital Status: Married  
Wife: Susan Jayne  
Daughter: Macara Marie, born 5 February 1992

***PRESENT POSITION***

1996 to present

Chairman, Research Department  
Naval Dental School

1994-1995

Research Administrator for Endodontics, Periodontics  
Oral Medicine, Oral Pathology, and Oral Surgery

Assistant Professor of Periodontics  
Research Department  
Naval Dental School

Adjunct Assistant Professor  
George Washington University  
Washington, DC

Course Director and Principal Lecturer  
NDS Oral and Written Scientific Communication, 16 hours

Staff Periodontist  
National Naval Dental Center  
Bethesda, Maryland

**PAST POSITIONS, continued**

1992 - 1993	Clinic Director and Staff Periodontist Third Dental Company, Third Dental Battalion Third Force Service Support Group United States Marine Corps, Okinawa, Japan
1988 - 1992	Staff Periodontist Naval Dental Center, San Francisco
1986 - 1988	Periodontics Resident Naval Dental School Bethesda, Maryland
1985 - 1986	Chief, Oral Diagnosis Department U.S. Naval Dental Clinic, Subic Bay Republic of the Philippines
1982 - 1985	Clinic Director and Assistant Dental Officer Naval Air Facility, Detroit Mount Clemens, Michigan
1980 - 1982	Assistant Dental Officer and Division Officer Dental Department U.S.S. Kitty Hawk, CV-63 San Diego, California
1979 - 1980	General Practice of Dentistry Resident Naval Hospital, Oakland, California

**EDUCATION AND TRAINING**

1986 - 1988	Periodontics Residency Naval Dental School Bethesda, Maryland Certificate, 1988
1979 - 1980	General Practice Residency Naval Hospital, Oakland, California Certificate, 1980
1976 - 1979	University of the Pacific School of Dentistry San Francisco, California Doctor of Dental Surgery, 1979
1972 - 1976	University of California, Berkeley Berkeley, California A.B. Zoology, 1976

## PROFESSIONAL LICENSE AND BOARDS

Diplomate, American Board of Periodontology, #965  
Licensed, State of California, #28365

## PUBLICATIONS

Ehrich, D., J. Lundgren, Dionne, R., Nicoll B., and Hutter, J.  
Comparison of oral triazolam, diazepam, and placebo as  
outpatient premedication for endodontic patients. *J Endodont* (in  
press)

Goodell, G., Mork, T., and Nicoll B. Linear dye penetration  
using calcium phosphate cement as an apical barrier. *J Endodont*  
(in press)

Anderegg C.R., Metzler D., Nicoll B.K.: "Gingiva thickness  
in guided tissue regeneration and associated recession at facial  
molar furcations." *J Periodontol* 1995;66:397-402.

Nicoll B.K. and Paul B.F.: "Periodontal Monographs."  
Naval Dental School, Bethesda, Maryland. 1988

Nicoll B., Mellonig J., Bosworth B.: "Comparison of  
surgical and nonsurgical therapy for periodontitis post-hygiene  
phase." *J Dent Res* 1988;67:212. (abstract)

## TEACHING EXPERIENCE

1993 - present

Assistant Professor of Periodontics  
Naval Dental School  
Bethesda, Maryland

1993 - present

Adjunct Assistant Professor  
George Washington University  
Washington, DC

1992

Adjunct Assistant Professor of Stomatology  
University of California, San Francisco  
School of Dentistry  
San Francisco, California

1990 - 1991

Adjunct Assistant Professor of Periodontics  
University of the Pacific  
School of Dentistry  
San Francisco, California

## LECTURE PRESENTATIONS

"Assessment of Risk Factors for Periodontitis"  
"Disease Indicators in Periodontitis"  
"A Motivational Approach to Oral Hygiene"

"An Overview of Antimicrobial Mouthrinses"

"Better Mousetraps for Oral Hygiene: New Horizons for the Electric Toothbrush"

Naval Dental School, Bethesda, Maryland, 1995 as part of the "Recruit to Retiree" Preventive Dentistry CE Course

"Oral Scientific Communication"

Naval Dental School, Bethesda, Maryland, 1995

"Disease Indicators and Risk Management in Periodontitis."

Naval Dental School, Bethesda, Maryland, 1994 and 1995 as part of Periodontics Short and Long Courses

"Organization for Technical Writing." Naval Dental School, Bethesda, Maryland, 1994

"Style and Other Stuff I Forgot Since English 1A."

Naval Dental School, Bethesda, Maryland, 1994

"Elements of the Scientific Paper."

Naval Dental School, Bethesda, Maryland, 1994.

"Diagnosis and Management of the Endodontic and Periodontic Lesion." Third Dental Battalion Continuing Education Study Group, Okinawa, Japan, 1993.

"Coronally-Positioned Flaps."

38th Parallel Dental Society, Seoul, Republic of Korea, 1993.

"Diagnosis and Management of Acute Periodontal Lesions."

Naval Hospital, Oakland, California, 1992.

"Analysis of Clinical Trials in Periodontics."

Naval Hospital, Oakland, California, 1990.

"Comparison of Surgical and Nonsurgical Therapy for Periodontitis Post-hygiene Phase." District 8 Meeting, American Academy of Periodontology, Washington, D.C., 1989.

"Comparison of Surgical and Nonsurgical Therapy for Periodontitis Post-hygiene Therapy." International Association for Dental Research, Montreal, Canada, 1988.

"Periodontal Considerations for Fixed Prosthetics." Naval Dental School Continuing Education Course in Periodontics, 1988.

"Comprehensive Examination and Diagnosis of Periodontal Diseases." Philippine Dental Association General Meeting, Manila, Republic of the Philippines, 1986.

### **HONORS AND AWARDS**

Navy Commendation Medal  
Third Dental Company, Third Dental Battalion  
Third Force Service Support Group  
United States Marine Corps, Okinawa, Japan, 1993.

Navy Commendation Medal  
Naval Dental Center, San Francisco, 1992.

Navy Achievement Medal  
Naval Dental Center, San Francisco, 1991.

Commanding Officer's Award for Excellence  
Naval Dental School  
National Naval Dental Center, 1987.

Humanitarian Service Medal (2 Awards)  
USS KITTY HAWK, CV-63, 1981.

Member, Tau Kappa Omega Honor Fraternity  
University of the Pacific, School of Dentistry  
San Francisco, California, 1978.

Member, Phi Beta Kappa Honor Fraternity  
University of California, Berkeley, 1975

### **PROFESSIONAL ORGANIZATIONS**

Diplomate, American Board of Periodontology  
1991 - present  
Member, American Academy of Periodontology  
1986 - present  
Treasurer, District Eight, American Academy of Periodontology  
1994 - present  
Member, American Dental Association  
1979-present  
Member, International Association for Dental Research  
1987 - present  
Secretary-Treasurer, Naval Dental School Faculty Forum  
Past-President, San Francisco Bay Area Armed Forces Dental  
Study Group, 1991  
Vice President, SFBAAFDSG, 1990  
Secretary-Treasurer, SFBAAFDSG, 1989

## CURRICULUM VITAE

### 1. PERSONAL INFORMATION

- 1.1. Glenn A. Miller, Ph.D.
- 1.2. Date and Place of Birth: January 19, 1948 Allentown, PA.
- 1.3. Citizenship: U.S.A.
- 1.4. Social Security Number: 188-38-8739:
- 1.5. Marital Status: Married, two children
- 1.6. Home Address and Telephone: 15833 Bradford Drive Laurel, MD  
20707 (301)317-0454
- 1.7. Fax Number: (301)292-6474
- 1.8. E-mail number: gmiller@btdacr.med.navy.mil
- 1.9. Office Address and Telephone: Research Department Naval Dental School National  
Naval Dental Center ; Bethesda, MD 20889-5602 (301)295-0460

### 2. EDUCATION

#### 2.1. Institution and Degrees:

- 2.1.1. 1969-1973 SYRACUSE UNIVERSITY; Syracuse, New York; Ph.D., Microbiology  
and Immunology
- 2.1.2. 1965-1969 MUHLENBERG COLLEGE; Allentown, Pennsylvania; B.S. Biology

#### 2.2. Postdoctoral:

- 2.2.1. 1973-1976 SCRIPPS CLINIC & RESEARCH FOUNDATION; La Jolla, California;  
Certificate in Immunopathology

#### 2.3. Other Significant Training:

- 2.3.1. 1993 : CATHOLIC UNIVERSITY OF AMERICA, Washington, D.C.; Certificate in In  
Situ Hybridization at the Center for Advanced Training
- 2.3.2. 1987: CATHOLIC UNIVERSITY OF AMERICA, Washington, D.C.; Certificate in DNA  
Methodology at the Center for Advanced Training
- 2.3.3. 1985: VIRGINIA COMMONWEALTH UNIVERSITY; Richmond, Virginia;  
biotechnology
- 2.3.4. 1981: HOOD COLLEGE; Frederick, Maryland; Certificate in Hybridoma and  
Monoclonal Antibody Methodology
- 2.3.5. 1978, 1980 : NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH; Jefferson,  
Arkansas; Training in Multiparameter Flow Cytometry.

#### 2.4. Honors and Awards:

- 2.4.1. 1994 - United States Navy Dental Corps and the Naval Dental School Civism  
Award for distinguished service and support of basic science and research  
education program at the Naval Dental School.
- 2.4.2. 1985 - A.H. Robins Commendation, Development of @BioCox
- 2.4.3. 1973 - the Dr. Alexander Gourevitch Memorial Award in Microbiology for the  
Outstanding Graduate Student of 1973. Syracuse University, Department of  
Biology
- 2.4.4. 1972-1973 - National Institutes of Health Postdoctoral Fellowship, Scripps  
Clinic and research Foundation, La Jolla, California.



- 2.4.5. 1972 to 1973 - Syracuse University Fellowship
- 2.4.6. 1969 to 1972 - National Institutes of Health Research Assstantship
- 2.4.7. 1969 - National Science Foundation Fellowship; New York State College of Forestry.

### 3. ACADEMIC APPOINTMENTS AND OTHER PROFESSIONAL EXPERIENCE

- 3.1. 1995-present : Johns Hopkins University; Baltimore, Maryland. Lecturer in Biochemistry, Part-time graduate studies program.
- 3.2. 1994 to present: Department of Microbiology and Immunology, George Washington University Medical School, Adjunct Professor
- 3.3. 1990 to present: NATIONAL NAVAL DENTAL CENTER; Bethesda, MD . Professor of Microbiology and Immunology, Naval Dental School
- 3.4. 1991 to present : GEO-CENTERS, INC.; Washington, D.C. ; Senior Scientist I and Group Manager III.
- 3.5. 1990 to 1991 : GEO-CENTERS, INC.; Washington, D.C. Senior Scientist II.
- 3.6. 1981-1990 : A.H. ROBINS COMPANY; Richmond, VA.; Group Manager, Microbiology and Immunology.
- 3.7. 1976 to 1990 : MEDICAL COLLEGE OF VIRGINIA OF VIRGINIA COMMONWEALTH UNIVERSITY; Richmond, VA. ;Adjunct Associate Professör, Department of Microbiology (1981-1992) . Assistant Professor, Department of Microbiology and Staff Member, Cancer Research Center for Periodontal Disease of the Medical College of Virginia (1978-1981)
- 3.8. 1977 to 1980: MATHEMATICS AND SCIENCE CENTER; Richmond, VA. Member of the Faculty.
- 3.9. 1980 to 1985: VIRGINIA STATE UNIVERSITY; Petersburg, VA. Adjunct Professor, Department of Life Sciences.
- 3.10. 1980 to 1981 W. ALTON JONES CELL SCIENCE CENTER; Lake Placid, NY. Member of the Faculty.

### 4. MEMBERSHIP IN SCIENTIFIC, HONORARY AND PROFESSIONAL SOCIETIES

- 4.1. The American Association of Immunologists
- 4.2. The Federation of American Societies for Experimental Biology
- 4.3. The American Association for Dental Research
- 4.4. The American Society for Microbiology
- 4.5. The Virginia Academy of Science
- 4.6. The New York Academy of Sciences

### 5. GRANT SUPPORT:

- 5.1. American Cancer Society Institutional Research Grant IN-105B, "Macrophage Heterogeneity in Tumor Immunity." Total allocation \$3,000 (October 1, 1976 - September 30, 1977). Principal Investigator.
- 5.2. Institutional Research Grant, supported by the Grants-In-Aid Program for Faculty of Virginia Commonwealth University. "Macrophage Heterogeneity." Total allocation \$4,000 (January 1, 1978 - December 31, 1978). Principal Investigator.
- 5.3. National Institutes of Health Research Grant No. 1 RO1 DE- 04398, "Severe Periodontitis: Immunologic Studies." Total allocation \$395,794 (May, 1976 - April, 1979). Co- investigator.

- 5.4. Institutional Travel Grant from the Cancer Center of the Medical College of Virginia, Virginia Commonwealth University to travel to the National Center for Toxicological Research in Jefferson, Arkansas to learn to operate the Beckman Elutriation Rotor and become acquainted with the techniques of Multiparameter Flow Cytometry. Total Allocation \$500 (Spring, 1978). Principal Investigator
- 5.5. Institutional Equipment Grant from the Cancer Center of the Medical College of Virginia of Virginia Commonwealth University for the purchase of cell culture apparatus. Total Allocation \$1,000 (1978). Principal Investigator
- 5.6. Institutional Research Grant, supported by the Grants-in-Aid Program for Faculty of Virginia Commonwealth University. "Use of Sophisticated Multiparameter Flow Cytometry to Study Macrophage Heterogeneity." Total Allocation \$1,557 (May 1, 1980 - April 30, 1981). Travel grant to spend two weeks at the National Center for Toxicological Research, Jefferson, Arkansas.
- 5.7. National Institutes of Health Research Grant No. 1 P50 DE05139, "Clinical Research Center for Periodontal Disease." Total Allocation \$1,996,000 (August, 1978 - July, 1983). Co-investigator.
- 5.8. National Institutes of Health Research Grant No. 1 T32 CA09036, "Macrophage Extrinsic Activity vs. Virus." Total Allocation \$180,209 (December 1, 1978 - November 30, 1981). Co-investigator.
- 5.9. American Cancer Society Research Grant IM-206, "Macrophage Heterogeneity in Tumor Immunity." Total Allocation \$80,209 (January 1, 1979 - December 31, 1980). Principal Investigator
- 5.10. Institutional Research Grant, supported by the Grants-in-Aid Program for Faculty of Virginia Commonwealth University. "Use of Sophisticated Multiparameter Flow Cytometry to Study Macrophage Heterogeneity." Total Allocation \$1,557 (May 1, 1980 - April 30, 1981). Travel grant to spend two weeks at the National Center for Toxicological Research, Jefferson, Arkansas.
- 5.11. National Institutes of Health Research Grant No. 1 RO1 AI 71423, "Regulation of the Immune Response by Persisting Antigen: Role of Follicular Antigen Binding Cells." Total Allocation \$116,527 (September 1, 1980 - August 31, 1983). Co-investigator.
- 5.12. American Cancer Society Research Grant No. IM-206A, "Macrophage Heterogeneity in Tumor Immunity." Total Allocation \$131,406 (January 1, 1981 - December 31, 1982). Principal Investigator.
- 5.13. National Institutes of Health MARC Fellowship, Total Allocation \$78,000 (1979 - 1982). Sponsor.
- 5.14. U.S. Navy Research and Development Command Individual Research Grant No. 61152N M00001.001-0063 "Evaluation of the Influence of Superantigens and Polyclonal B-cell Activators in Periodontal Diseases". Total Allocation \$345,000 (October 1, 1992 - September 28, 1995). Principal Investigator.

## 6. SCHOLARLY, RESEARCH OR ADMINISTRATIVE EXPERIENCE

### 6.1. Presentation of Special Seminars or Lectures :

- 6.1.1. Department of Microbiology, University of Kansas, Lawrence, Kansas; October, 1975. Invited seminar speaker
- 6.1.2. Department of Microbiology, University of Oklahoma, Norman, Oklahoma, October, 1975. Invited seminar speaker
- 6.1.3. Department of Medicine, Medical College of Virginia of Virginia Commonwealth University, Richmond, VA.; February; 1976. Invited seminar speaker

- 6.1.4. Department of Microbiology, Medical College of Virginia of Virginia Commonwealth University, Richmond, Virginia.; October, 1975. Invited seminar speaker
- 6.1.5. Department of Biology, Muhlenberg College, Allentown, Pennsylvania; April, 1976. Guest lecturer
- 6.1.6. National Center for Toxicological Research, Jefferson, Arkansas; March, 1976. Invited seminar speaker
- 6.1.7. Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California; May, 1976. Invited seminar speaker
- 6.1.8. Biological Seminar, Medical College of Virginia, Richmond, Virginia; January, 1977. Invited speaker Department of Biology, Virginia Commonwealth University, Richmond, Virginia; March, 1977. Invited speaker
- 6.1.9. National Center for Toxicological Research, Jefferson, Arkansas; March, 1978. Invited seminar speaker
- 6.1.10. Department of Biology, Virginia Commonwealth University, Richmond, Virginia; March, 1978. Invited speaker
- 6.1.11. WWBT News, Richmond, Virginia; December, 1978. Invited speaker
- 6.1.12. Science Department, John F. Kennedy High School, Richmond, Virginia; February, 1978. Guest lecturer
- 6.1.13. Animal Parasitology Division, United States Department of Agriculture, Beltsville, Maryland; October, 1979. Invited seminar speaker
- 6.1.14. Division of Basic Sciences, Marquette University School of Dentistry, Milwaukee, Wisconsin; February, 1980. Guest speaker
- 6.1.15. Department of Biology, Muhlenberg College, Allentown, Pennsylvania; April, 1980. Guest speaker
- 6.1.16. Division of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee; August, 1980. Guest seminar
- 6.1.17. Molecular Biology Department, A.H. Robins Co., Richmond, Virginia; May, 1981. Guest speaker
- 6.1.18. Pharmacology Division, A.H. Robins Co., Richmond, Virginia; October, 1981. Guest speaker
- 6.1.19. Department of Microbiology, Medical College of Virginia of Virginia Commonwealth University; September, 1985. Invited seminar speaker
- 6.1.20. Genex Corporation, Gaithersburg, Maryland; September, 1986. Guest speaker
- 6.1.21. Genex Corporation, Gaithersburg, Maryland; October, 1987. Guest speaker
- 6.1.22. Department of Veterinary Medicine, Perdue Farms, Inc. Maryland; September, 1988. Guest speaker
- 6.1.23. Fort Dodge Laboratories, Fort Dodge, Kansas; November, 1988. Guest speaker
- 6.1.24. Research and Development Division, Syntro Corporation, San Diego, California; August, 1988. Invited seminar speaker
- 6.1.25. Research and Development Division, Grand Island Biological Corporation, Grand Island, New York; February, 1989. Invited seminar speaker
- 6.1.26. Department of Microbiology, Alcon Laboratories, Fort Worth, Texas; May, 1990. Guest speaker
- 6.1.27. Research Department, National Naval Dental Center, Bethesda, Maryland; June, 1990. Invited seminar speaker
- 6.1.28. Department of Bacteriology and Botany, Auburn University, Auburn, Alabama; August, 1990. Invited seminar speaker

- 6.1.29. Epidemiology and Oral Diseases Prevention Program, National Institutes of Dental Research/ National Institutes of Health, Bethesda, MD, 1992. Invited seminar speaker
- 6.1.30. Naval Dental Research Institute, Great Lakes, Illinois; 1991, 1994. Seminar Speaker
- 6.1.31. Department of Microbiology and Immunology, George Washington University Medical School, Washington, D.C. ; 1994. Invited Seminar Speaker
- 6.1.32. Portsmouth Naval Hospital, Portsmouth, Virginia; 1994. Invited Seminar Speaker

## 6.2. Participation In Special Courses

- 6.2.1. Presented a 10-week Microbiology Course for high school students as part of the Mathematics and Science Center's Saturday Morning Explorers Program. Mathematics and Science Center, Glen Allen, Virginia. 1977-1978, 1978-1979, 1979-1980.
- 6.2.2. Mini-sabbatical at the National Center for Toxicological Research in Jefferson, Arkansas to become acquainted with techniques of counter-flow centrifugation (elutriation) and techniques involved in multiparameter flow cytometry. Spring 1978.
- 6.2.3. Mini-sabbatical at the National Center for Toxicological Research in Jefferson Arkansas working with the Coulter Epics V Flow Cytometry System and MDADS. Summer 1980.
- 6.2.4. Participated as Adjunct Faculty in the course "Culture of Macrophages and Mononuclear Phagocytes" at the W. Alton Jones Cell Science Center, Lake Placid, New York, October, 1980.
- 6.2.5. Presented a 16-week course entitled "Basic, Clinical and Applied Immunology" in the Research and Development Division, A.H. Robins Co., Richmond, Virginia, 1982.

## 6.3. Major Committees And Review Committees

- 6.3.1. University Radiation Safety Committee, Virginia Commonwealth University; 1978-1981
- 6.3.2. Academic Performance Committee of the School of Dentistry, Virginia Commonwealth University; 1979-1990.
- 6.3.3. NCI, NIH, Biological response Modifiers Program, Decision Network Committee, 1983
- 6.3.4. Special Reviewer - Journal of the Reticuloendothelial Society, 1982, 1985
- 6.3.5. Special Reviewer - Journal of Pharmacology, 1988
- 6.3.6. Recombinant DNA Safety Committee, A.H. Co., Richmond, Virginia; 1986-1990
- 6.3.7. Institutional Biosafety Committee, A.H. Robins Co., Richmond, Virginia; 1981-1990
- 6.3.8. Special Reviewer - Naval Research and Development Command, Bethesda, Maryland

## BIBLIOGRAPHY

### Articles:

1. Miller, G. A. and R. W. Jackson. 1973. The effect of a streptococcus pyogenes teichoic acid on the immune response of mice. *J. Immunol.* 110:148.
2. Miller, G. A., J. Urban, and R. W. Jackson. 1976. Effects of streptococcal lipoteichoic acid on host responses in mice. *Infect. Imm.* 13:1408.
3. Miller, G. A. and J. D. Feldman. 1976. Genetic role of rat macrophage cytotoxicity against tumor. *Int. J. Cancer* 18:168.
4. Veit, B. C., J. M. Jones, G. A. Miller, and J. D. Feldman. 1976. Genetic association of the humoral and cellular immune responses of rats to Moloney sarcoma. *Int. J. Cancer* 19:97.
5. Miller, G. A. and J. D. Feldman. 1977. Effect of macrophages and antibodies on *in vivo* growth of Moloney sarcoma in the rat. *J. Immunol.* 119:1445.
6. Ranney, R., J. Tew, G. Miller, H. Welshimer, K. Palcanis, W. Jordan, and P. Rice. 1979. Human antibody response and blastogenic response of human peripheral blood lymphocytes to oral bacteria in individuals with severe periodontitis. Fourth International Conference on Periodontal Research, Stockholm, Sweden. *J. Periodontal. Res.* 14:257.
7. Tew, J. G., T. E. Mandel, and G. A. Miller. 1979. Immune retention: immunological requirements for maintaining an easily degradable antigen *in vivo*. *Aust. J. Exp. Biol. Med. Science* 57 (pt. 4):401.
8. Greene, E. J., J. G. Tew, and G. A. Miller. 1980. Effects of specific antigen and specific antibody on the kinetics of *in vitro* antibody production. *Cell. Immunol.* 53:307.
9. Campbell, M. W., M. M. Sholley, and G. A. Miller. 1980. Macrophage heterogeneity in tumor resistance: cytostatic and cytotoxic activity of *Corynebacterium parvum* activated and proteose peptone elicited rat macrophages against Moloney sarcoma tumor cells. *Cell. Immunol.* 50:163.
10. Miller, G. A., M. W. Campbell, and J. L. Hudson. 1980. Separation of rat peritoneal macrophages into functionally distinct subclasses by centrifugal elutriation. *J. Reticuloendothel. Soc.* 27:167.
11. Phipps, R. P., J. G. Tew, and G. A. Miller. 1980. A murine model for spontaneous induction and feedback regulation of specific antibody synthesis in murine lymph node cells adoptively transferred without *in vivo* incubation. *Imm. Comm.* 9(1):55.

12. Smith, S., P. H. Bick, G. A. Miller, R. R. Ranney, P. L. Rice, J. H. Lalor, and J. G. Tew. 1980. Polyclonal B-cell activation: severe periodontal disease in young adults. *Clin. Immun. Immunopath.* 16:354.
13. Donaldson, S. L., G. A. Miller, P. L. Rice, R. R. Ranney, and J. G. Tew. 1981. The maintenance of B-cell and T-cell function in frozen and stored lymphocytes. *J. Clin. Imm.* 7:106.
14. Morahan, P. S. and G. A. Miller. 1981. Heterogeneity of *Corynebacterium parvum* elicited macrophages. First International Conference on Macrophage Heterogeneity. Bade, Vienna. *Proc. First Int. Conf. Macrophage Heterogeneity* 1:18.
15. Tew, J. G., G. A. Miller, E. J. Greene, P. L. Rice, W. P. Jordan, K. G. Palcanis, and R. R. Ranney. 1981. Immunological studies of young adults with severe periodontitis, II. Cellular factors. *J. Periodontal. Res.* 16:403.
16. Bick, P. H., B. Carpenter, L. V. Holdeman, G. A. Miller, R. R. Ranney, K. G. Palcanis, and J. G. Tew. 1981. Polyclonal B-cell activation induced by extracts of gram-negative bacteria isolated from periodontally diseased sites. *Infect. Imm.* 34:43.
17. Miller, G. A. and P. S. Morahan. 1982. Functional and biochemical heterogeneity among subpopulations of rat and mouse peritoneal macrophages. *J. Reticuloendothel. Soc.* 32:111.
18. Miller, G. A., R. C. Clark, and E. J. Jesse. 1986. Production of monoclonal antibodies to salinomycin. *Hybridoma* 5:353.
19. Miller, G. A., B. S. Bhogal, R. McCandliss, R. L. Strausberg, E. J. Jessee, A. C. Anderson, C. K. Fuchs, and S. Strausberg. 1989. Characterization and vaccine potential of a novel recombinant coccidiosis antigen. *Infect. Imm.* 57:2014.
20. Miller, G., B. Bhogal, A. Anderson, E. Jessee, R. McCandliss, M. Likel, J. Strasser, R. Strausberg, and S. Strausberg. 1989. Application of a novel recombinant *Eimeria tenella* antigen in a vaccine to protect broiler chickens from coccidiosis. *Recent Adv. Avian Immunol. Res.* 307:117-130. (Eds), Bhogal B.s. and G. Koch, Alan R. Liss.
21. Bhogal, B., G. Miller, E. Jessee, A. Anderson, R. Strausberg, R. McCandliss, and S. Strausberg. 1989. Vaccination of chickens with recombinant *E. tenella* antigen alone or in combination with a subclinical exposure induces cross protective immunity against coccidiosis. *Recent Adv. Avian Immunol. Res.* 307:131-148. (Eds), Bhogal B.s. and G. Koch, Alan R. Liss.



14. Miller, G. A. 1980. Functional heterogeneity of *Corynebacterium parvum* activated rat macrophages. *Fed. Proceed.* 39:1275.(Abstract)
15. Miller, G. A. and M. M. Sholley. 1980. Heterogeneity of *Corynebacterium parvum* activated rat macrophages. *Fourth International Conference of Immunology, Paris, France* 8.19:11.(Abstract)
16. Morahan, P. S. and G. A. Miller. 1980. Heterogeneity of *Corynebacterium parvum* elicited macrophages. *Proceedings of the Fourth International Conference of Immunology, Paris, France* 8.19:12.(Abstract)
17. Phipps, D. C. and G. A. Miller. 1981. Stimulation of rat T-lymphocytes by subpopulations of antigen pulsed macrophages. *Federation Proceedings* 40:1096.(Abstract)
18. Lariscy, L., R. Ranney, and G. A. Miller. 1982. Role of the monocyte in polyclonal B-cell activation. *Int. Assoc. Dent. Res. Abs. New Orleans, La.*:(Abstract)
19. Miller, G. A., S. M. Weik, and R. W. Tankersley. 1983. Production of a monoclonal antibody to chicken IgA and its use in an enzyme linked immunoassay for detection of antibody to bursal virus. *Proc. 5th Int. Congress of Immunol.* (Abstract)
20. Miller, G. A. and D. C. Phipps. 1983. Induction of T-cell proliferation by presentation of trinitrophenylalbumin on subpopulations of proteose peptone elicited or *Corynebacterium parvum* activated rat macrophages. *Proc. 5th Int. Congress of Immunol.* (Abstract)
21. Clark, R. C. and G. A. Miller. 1984. Production of monoclonal antibodies to salinomycin and their use in the development of a quantitative enzyme linked immunosorbent assay. *Third Annual Congress for Hybridoma Research. Hybridoma* 3:81.(Abstract)
22. Bhogal, B., G. Miller, E. Jessee, A. Anderson, R. Strausberg, R. McCandliss, and S. Strausberg. 1988. Vaccination of chickens with recombinant *E. tenella* antigen alone or in combination with a subclinical exposure induced cross protective immunity against coccidiosis. *Avian Immunology Research Group Meeting, Lelystad, The Netherlands* (Abstract)
23. Miller, G., B. Bhogal, A. Anderson, E. Jessee, R. McCandliss, R. Strausberg, and S. Strausberg. 1988. Application of a novel recombinant antigen in a vaccine to protect broiler chickens from coccidiosis. *Avian Immunology Research Group Meeting, Lelystad, The Netherlands* (Abstract)
24. Miller, G. A., A. Anderson, and B. Bhogal. 1988. Enrichment of parasite-specific T cells from splenic lymphocytes of immune chickens by percoll gradients. *FASEB Journal* 2:A882:(Abstract)

25. Meiers, J.C., T.E. Griffo and G.A. Miller. 1992. Antimicrobial activity of dentin bonding systems and glass ionomers. *AADR Annual Meeting Abstract: (JDR) No. 1639* (Abstract)
26. Miller, G.A. and S. White. 1992. Cytokine production by heterogeneous rat macrophages. *Proceedings of the Eighth International Congress of Immunology*: Abs. No. 24, p.142. (Abstract)
27. Melvin, W., D. Assad, G.A. Miller, M. Gher, L. Simonson, and A. York. 1993. Comparison of DNA hybridization probe and ELISA microbial analysis. *Journal of Dental Research* No. 2443, p. 409. (Abstract)
28. Getka, T., D. Alexander, W. Parker, and G.A. Miller. 1994. Immunomodulation by components of bacteria associated with adult periodontitis. Abstract No. 1212. *Journal of Dental Research* 73: 253. (Abstract)
29. Barco, C.T, G.A. Miller, and J. Schwartz. 1994. Effect of growth factors on periodontal, gingival, and tumor fibroblasts. *Journal of Dental Research* (Abstract) IADR 13th International Conference on Oral Biology., Victoria, BC, Canada.
30. Hickey, M. and G. A. Miller 1994. Functional response of frozen and stored human lymphocytes to superantigens. Abstract No. 819. *Journal of Dental Research* 73:253 (Abstract).
31. Miller, G. A., T. DeMayo and J. Hutter. 1994. Production of interleukin-1 by polymorphonuclear leukocytes present in inflammatory periradicular tissues. *Experimental Biology* 94 : 1200 (Abstract)
32. Perez M, D. Assad, and G.A. Miller. 1994. Incidence of systemic bacteremia with e-PTFE membrane use in periodontal surgery-. A pilot study. Abstract No. 1474. *Journal of Dental Research* 1994;73:286.(Abstract)
33. DeMayo T, Hutter J, Miller GA. Production of interleukin-1 by polymorphonuclear leukocytes present in inflammatory periradicular tissues. *Journal of Dental Research* 1994;(Abstract)
34. Canaan, T, M.Hickey, A.W. York, and G.A. Miller. 1995. Functional and autologous immune responses of cryopreserved human lymphocytes. Abstract No. 910, *Journal of Dental Research*. 74:125.
35. Miller, G.A. and D.C.Alexander. 1995. Evidence for superantigen production by bacteria associated with adult periodontitis. Abstract No. 906, *Journal of Dental Research* 74:73.



36. Miller, G.A. M.M. D'Alesandro, Xinbin,Gu, and S. Diehl. 1995. Evidence for superantigen production by bacteria associated with adult periodontitis. Abstract No. 1884. **Proceedings of the 9th International Convocation of Immunology**, San Francisco, CA.
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# Immunomodulatory and Superantigen Activities of Bacteria Associated With Adult Periodontitis

Thu P. Getka,\* David C.C. Alexander,\* William B. Parker,\* and Glenn A. Miller\*\*

IMMUNE DYSFUNCTIONS ARE FREQUENTLY associated with chronic inflammatory diseases. Several investigators have reported that patients with severe periodontitis show reduced or negligible levels of proliferative responses of peripheral blood and gingival lymphocytes to periodontopathic organisms. The purpose of this study was to evaluate the influences of products from *Porphyromonas gingivalis* (*P. gingivalis*) and *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) on lymphocytes obtained from periodontally diseased and non-diseased individuals in order to extend our understanding of the possible role of such bacterial components as immune modulators. Pooled cultures of either *P. gingivalis* or *A. actinomycetemcomitans* were disrupted using glass beads in a bead mill to prepare whole cell homogenates. These homogenates were then co-cultured with human peripheral blood mononuclear cells (PBMC) and known lymphocyte stimulators. Cultures were pulsed with tritiated thymidine, harvested, and radio label incorporation was determined. Responses to toxic shock syndrome toxin-1 (TSST-1) and pokeweed mitogen (PWM) were inhibited at high concentrations of bacterial homogenate. However, as the concentration was reduced, responses induced by PWM were restored while TSST-1 induced responses remained inhibited. Such results suggest that *P. gingivalis* and *A. actinomycetemcomitans* contain potent immunosuppressants with differential influences on lymphocyte population. These effects on B- and T-cells are independent of periodontal disease status and appear to exert their influence through non-toxic mechanisms. In addition, work currently underway presently indicates that obligate oral anaerobic bacteria such as *P. gingivalis* produce substances with some of the characteristics of superantigens. *J Periodontol* 1996;67:909-917.

**Key Words:** Immune response; immune system deficiency; lymphocytes; periodontitis/complications; periodontitis/microbiology.

Bacteria have been known to develop mechanisms to evade or neutralize the host immune defense at the innate and acquired levels. Immunosuppressive factors include enzymes, toxins, cell wall components, and metabolites.<sup>1</sup> Many authors have suggested an interaction between the immunosuppressive properties of oral pathogens and the host's immune cells as a prerequisite for the development of periodontal disease.<sup>2-4</sup> Shenker tested several suspected periodontal pathogens for immunosuppressive potential.<sup>5</sup>

He found immunosuppression to be widely distributed among several *Bacteroides* species, *Treponema denticola*, *Fusobacterium nucleatum*, and *A. actinomycetemcomitans*. *A. actinomycetemcomitans* appeared to have the strongest relative inhibition as assessed by a comparison of the dose of crude sonic extract resulting in 50% reduction in lymphocyte activation. Ivanyi and Lehner studied the effects of the lymphocyte response when exposed to various oral bacterial components. They reported increased lymphocyte responses in patients with gingivitis and mild periodontitis and depressed responses in patients with severe periodontitis.<sup>5</sup> Other studies have yielded similar observations indicating increased lymphocyte response in periodontitis patients and minimal to no response in healthy patients.<sup>6-8</sup>

Based on these studies, it was postulated that specific

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sensitization of the lymphocyte occurs in patients with gingivitis and mild periodontitis with serum factor enhancing the blastogenic response. The lack of response was attributed to a "serum inhibitor" carried by severe periodontitis patients which blocked the blastogenic response.<sup>4</sup> Later studies failed to find a correlation between lymphoblastic response and the severity of the disease.<sup>9-14</sup>

During studies focused on an understanding of the immunomodulator activity associated with periodontal pathogens, we became intrigued with an indication that immunosuppression and immunoenhancing activities were present simultaneously in bacterial homogenates. In this report we suggest that B-cells and T-cells are influenced to significantly different extents when co-cultured in vitro with bacterial preparations and that such responses are not influenced by the periodontal health of the subjects.

## MATERIALS AND METHODS

### Subjects

The investigation was approved by the Committee for the Protection of Human Subjects at the National Naval Medical Center. Six patients with advanced periodontitis and 26 healthy controls were studied. The advanced periodontitis group exhibited > 5 mm loss of attachment and bleeding upon probing in at least 4 sextants. All subjects had numerous sites with radiographic evidence of alveolar bone resorption. The control group had no history of periodontitis, and no clinical or radiographic features of gingivitis or periodontitis at the time of examination. No probing depths > 3 mm were noted. All subjects were over 35 years of age, in good health, and with no history of systemic antibiotics in the previous 3 months. Due to the limited number of PBMCs recovered from each subject, not all individuals were utilized in every study.

### Preparation of Mononuclear Cells

Heparinized venous blood (50 to 100 ml) was diluted with Hank's balanced salt solution (HBSS), layered over Ficoll-Hypaque,<sup>8</sup> and centrifuged for 30 minutes at 900 × g at 18 to 20°C, with no brake. Mononuclear cells were harvested from the Ficoll-Hypaque interface, washed twice, and resuspended in RPMI-1640 and 10% AB<sup>+</sup> plasma. Cell viability was determined by trypan blue exclusion and phase contrast microscopy. Cells were counted in a hemocytometer and prepared at the appropriate concentration of viable cells per ml.

### Bacterial Extracts

*Porphyromonas gingivalis* (*P. gingivalis*; ATCC 33.277) and *A. actinomycetemcomitans* strain Y4 were obtained from the collection of the Anaerobe Laboratory of Vir-

ginia Polytechnic Institute and State University, Blacksburg. Bacteria were grown under anaerobic conditions in Wilkins-Chalgren broth<sup>15</sup> supplemented with 0.3 mg/ml menadione, and at 5 mg/ml hemin. Bacteria were washed and suspended in 1 part glycerol to 2 parts phosphate-buffered saline at approximately 0.1 g of cells per ml. The suspensions were stored at -20°C until processed. Bacterial extracts were thawed, the cells were washed in phosphate-buffered saline, resuspended, and disrupted using glass beads in a bead mill.<sup>1</sup> The degree of disruption was assessed by phase contrast microscopy. In specific instances insoluble wall material was removed by centrifugation and the soluble extract recovered. Bacterial protein concentration was determined using the BioRad Bradford assay.<sup>16</sup>

### Lymphocyte Stimulators and Assays

Pokeweed mitogen (PWM), toxic shock syndrome toxin-1 (TSST-1), staphylococcus enterotoxin A and B (SEA, SEB), and Concanavalin A (Con A),<sup>17</sup> were used to stimulate various cultures in the blast-transformation procedure. For the assay, PBMC were suspended in RPMI 1640 without antibiotic supplementation and aliquoted into round-bottom microtiter plates. Each well received 200 µl of cell suspension containing  $2 \times 10^5$  PBMC. After the specified incubation period in a 37°C humidified environment containing 5% CO<sub>2</sub>, cells were pulsed with [methyl-<sup>3</sup>H]thymidine (1 mCi/well, 40-60 Ci/mmol). Cells were harvested 24 hours later using a PHD multiple automatic sample harvester.<sup>18</sup> Radioactivity was determined in a liquid scintillation counter.<sup>19</sup> the individual assays were done at least in triplicate.

### Enzyme Treatment of Bacterial Extracts

Trypsin treatment was carried out according to the method of Smith.<sup>15</sup> Chymotrypsin, endoproteinase, and pepsin digestions were done according to procedures detailed by Allen<sup>16</sup> and Wilkinson.<sup>17</sup> In general, various bacterial homogenates in appropriate buffers and at optimal enzyme concentrations were incubated at 37°C for 24 hours. Excess enzyme was removed utilizing multiple passes over affinity columns with antibody to chymotrypsin and trypsin obtained from Synbiotics Corp. San Diego and antisera to endoproteinase and pepsin produced at A.H. Robins Inc., Richmond, VA. Affinity columns were constructed employing cyanogen bromide activated sepharose 4B following manufacturer's instructions.<sup>3</sup> Lack of residual enzyme in treated solutions was assessed by radial im-

<sup>8</sup>Pharmacia Fine Chemicals, Piscataway, NJ.

<sup>15</sup>Oxoid Ltd., Basingstoke, Hampshire, UK.

<sup>16</sup>Mini-Bead Beater, Biospec Products, Bartlesville, OK.

<sup>17</sup>BioRad Laboratories Life Science Group, Melville, NY.

<sup>18</sup>Sigma Chemical Co., St. Louis, MO.

<sup>19</sup>Cambridge Technology, Inc., Watertown, MA.

<sup>20</sup>Beckman LS6500, Beckman Instruments, Fullerton, CA.

munodiffusion procedures sensitive to 10 mcg/ml. The same antibody reagents used for preparation of the immunoabsorbent columns were used in the immunodiffusion procedures. Enzymes\*\* consisted of chymotrypsin (EC 3.4.21.1), endoproteinase Arg-C (EC 3.4.21.40), pepsin (EC 3.4.23.1), and trypsin (EC 3.4.21.4).

### Antibodies

Antibodies with specificity to antigens of the HLA class II DR histocompatibility locus and HLA-B were obtained from Cappel Laboratories (Malvern, PA). Antibody to CD3 was obtained from R&D Systems (Minneapolis, MN).

### Preparation of Lymphocyte Sub-Populations

Purified T-cell populations were prepared from selected PBMC suspensions by utilizing commercial T-cell enrichment columns as provided by R&D Systems. In general, an antibody cocktail was added to preparations of PBMCs to deplete cell populations of no interest (B-cells, monocytes, NK cells, and CD8+ T-cells). PBMC were passed through an appropriate column and enriched CD4+ cells were recovered in the effluent. In some instances populations of peripheral blood monocytes were prepared after isolation of monocytes by adherence to rat tail collagen coated tissue culture plates and then recovering them after collagenase treatment.<sup>15</sup> Monocytes were counted and added to T-cell preparations as indicated in results. Evaluation of the level of efficacy of cell removal was determined by fluorescence microscopy. Enriched T-cells and monocytes were always greater than 95%. B-cell contamination was always less than 1%.

### IL-2 Assay

IL-2 was assayed in cell culture supernatants utilizing quantitative enzyme linked immunosorbent assay kits purchased from R&D Systems (Minneapolis, MN). Assays were done according to the manufacturer's instructions supplied with the kits.

### Statistical Evaluations

Among groups, statistical significances were determined by Student *t* test for differences in means. Probability of equal to or less than 0.05 was considered significant.

### RESULTS

Homogenates from *P. gingivalis* and *A. actinomycetemcomitans* increased tritiated thymidine (<sup>3</sup>H-TdR) incorporation into PBMC. The highest mean level of stimulation was seen with *A. actinomycetemcomitans* homogenate in all individuals tested regardless of the periodontal status (6 periodontally diseased and 10 non-diseased individuals). Individual lymphocyte responses to various bacterial homogenate concentrations were consistently lower at elevated concentrations. Representative response

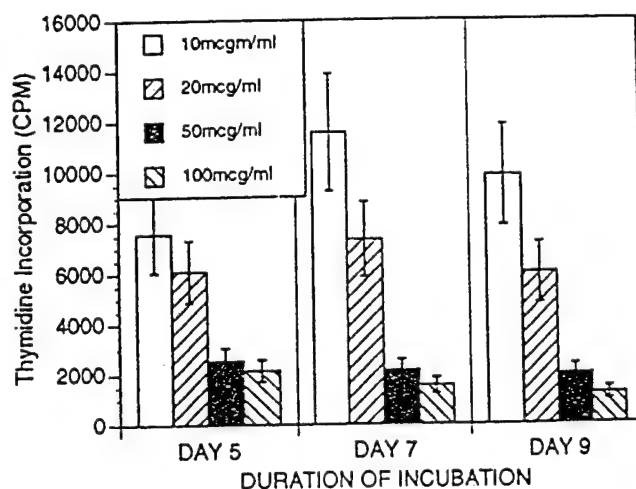


Figure 1. *A. actinomycetemcomitans* strain Y4 antigen induced stimulation of proliferation of PBMC from a non-diseased individual. Data represent the mean and standard deviation of four replicate cultures at each bacterial homogenate and incubation time. Thymidine incorporation in the absence of bacterial homogenate was  $375 \pm 85$  cpm. All responses are significantly different from background at  $P < 0.05$  or better using the Student *t*-test.

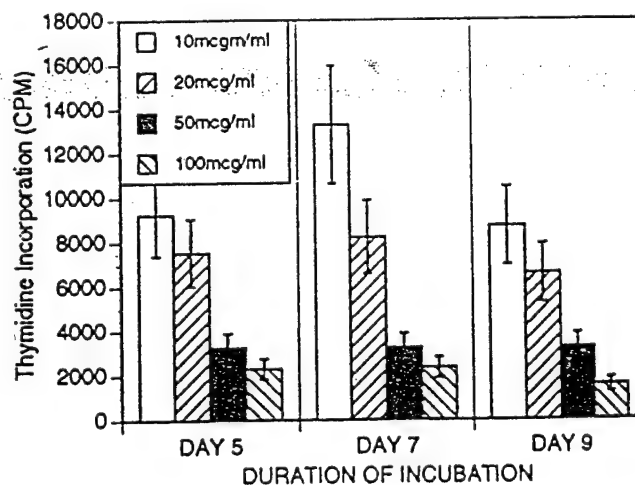


Figure 2. *A. actinomycetemcomitans* strain Y4 antigen-induced stimulation of proliferation of PBMC from a periodontally diseased individual. Data represent the mean and standard deviation of four replicate cultures at each bacterial homogenate and incubation time. Thymidine incorporation in the absence of bacterial homogenate was  $527 \pm 126$  cpm. All responses are significantly different from background at  $P < 0.05$  or better using the Student *t*-test.

patterns from a single individual are shown in Figures 1 and 2. Table 1 shows cell viability determined at various bacterial homogenate concentrations. No significant differences in percent viability were noted at 0, 10, 20, 50, and 100 mcg/ml. The percent of viable cells decreased steadily from day 4 to day 9 independent of whether homogenates were present. Assessment of viability of cells to *P. gingivalis* homogenates yielded essentially the same results. Data presented in Table 2 show results from 10

Table 1. Assessment of Cell Viability With and Without *A. actinomycetemcomitans* Homogenate at Various Concentrations and Incubation Periods

Homogenate (mcg/ml)	Incubation (day)			
	4	5	7	9
0	77.4 $\pm$ 7.64	73.6 $\pm$ 5.32	67.4 $\pm$ 7.09	60.6 $\pm$ 2.70
10	83.0 $\pm$ 5.70	72.6 $\pm$ 2.30	70.4 $\pm$ 2.51	59.4 $\pm$ 3.36
20	69.8 $\pm$ 8.20	72.0 $\pm$ 6.82	67.8 $\pm$ 5.54	58.2 $\pm$ 3.27
50	71.8 $\pm$ 9.31	67.0 $\pm$ 6.93	65.6 $\pm$ 6.43	59.6 $\pm$ 4.77
100	74.8 $\pm$ 9.83	74.6 $\pm$ 8.32	69.4 $\pm$ 3.97	64.0 $\pm$ 3.74

Results expressed as mean percent viability of 5 individuals  $\pm$  standard deviation. No significant differences (Student *t* test) in cell viability are observed when each value for a homogenate concentration was compared to the value obtained in the absence of homogenate tested with cells incubated for the same time period.

Table 2. Stimulation of PBMC From Healthy and Periodontally Diseased Individuals with *P. gingivalis* Homogenate

Homogenate (mcg/ml)	Percent of Maximal Response	
	Non-Diseased (n = 10)	Diseased (n = 6)
10	100 (62.5 $\pm$ 4.56)	100 (60.6 $\pm$ 5.24)
20	83 $\pm$ 13 (72.0 $\pm$ 6.32)	87 $\pm$ 14 (67.9 $\pm$ 6.23)
50	47 $\pm$ 11 (62.3 $\pm$ 5.32)	53 $\pm$ 13 (62.8 $\pm$ 5.23)
100	32 $\pm$ 12 (64.7 $\pm$ 6.81)	38 $\pm$ 18 (79.5 $\pm$ 3.20)

Results are expressed as a mean percent of the radiothymidine incorporation into PBMC  $\pm$  standard deviation. Data are normalized as a percent of the level of incorporation achieved for each individual after culturing PBMCs with 10 mcg/ml of the homogenate in order to compensate for the individual variation in absolute radiothymidine incorporation which ranged from 2374 to 14,740  $\pm$  4247 CPM for the non-diseased individuals and 3256 to 16,458  $\pm$  5334 CPM for diseased individuals. Italic values indicate significant differences (Student *t*-test,  $P < 0.05$ ) when compared to responses achieved with 10 mcg/ml homogenate. Bracketed values indicate the mean percent viability of cells in culture  $\pm$  standard deviation for cells cultured 5 days.

non-diseased and 6 diseased individuals normalized to percent of the maximal response generated at 10 mcg/ml homogenate. This was done to compensate for the individual variation in absolute levels of radiothymidine incorporation for each subject. The percent of maximal response decreased with increased bacterial homogenate for PBMCs from both groups.

In an effort to determine if immunomodulatory activity is involved, experiments were performed to study the inhibitory effects of bacterial extracts on lymphocyte proliferation when co-cultured with known stimulators of B-cells and T-cells. PWM was used as a T-cell dependent B-cell stimulator and the superantigen TSST-1 was used as a T-cell stimulator. Typical results from a co-culture experiment utilizing *A. actinomycetemcomitans* strain Y4 as the source of bacterial homogenate and PBMC from a non-diseased individual are shown in Figure 3. Both the TSST-1 and PWM induced responses were inhibited when co-cultured for 4 days. However, TSST-1 induced T-cell responses were inhibited to a greater extent than PWM induced B-cell responses. Inhibition of the TSST-1 induced response was significant ( $P < 0.05$ ) at 5 mcg/ml whereas inhibition of the PWM induced response was significant ( $P < 0.05$ ) at 25 mcg/ml.

*P. gingivalis* homogenates showed similar results when

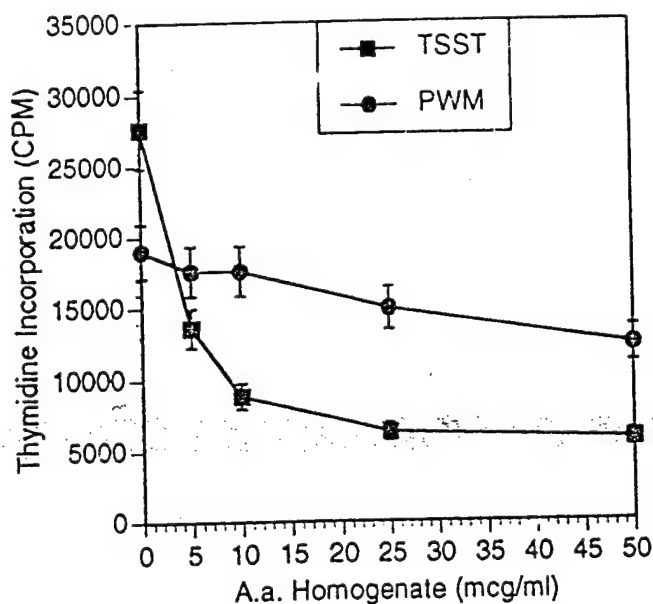


Figure 3. Proliferation of PBMC from a non-diseased individual cocultured with either TSST-1 or PWM and *A. actinomycetemcomitans* strain Y4 homogenates. Data represent the mean and standard deviation of four replicate cultures cultured for 4 days. Inhibition of the responses became significant for the TSST-1 co-culture at 5 mcg/ml whereas inhibition of the PWM response did not become significant until a level of 25 mcg/ml was reached.

lymphocytes were co-cultured with either TSST-1 or PWM. Typical results using PBMC from a non-diseased subject are shown in Figure 4. Inhibition of proliferation with TSST-1 was significant ( $P < 0.05$ ) at 50 mcg/ml after 6 days in culture whereas inhibition of proliferation with PWM became significant at 300 mcg/ml.

Since individual responses to PWM and TSST-1 differ, data from studies involving 10 non-diseased and 6 diseased individuals were normalized to percent of maximal responses under conditions of peak stimulation and response times. Homogenate concentrations were selected to result in 20% of maximal stimulatory levels (also as determined by  $^3\text{H}$ -TdR incorporation) when cultured with PBMC alone to ensure limited cell stimulation by bacterial components. It is clear that while responses to TSST-1 were significantly suppressed, no effect was ob-



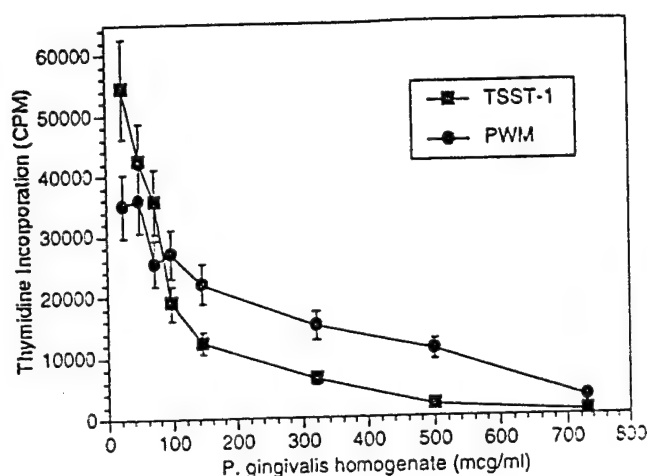


Figure 4. Proliferation of PBMC from a non-diseased individual cocultured with TSST-1 or PWM and *P. gingivalis* homogenates. Data represent the mean and standard deviation of four replicate cultures cultured for 6 days. Inhibition of the responses became significant at 50 mcg/ml when cells were stimulated with TSST-1 but required 300 mcg/ml when stimulated with PWM. The mean incorporation of  $^3\text{H}$ -TdR after TSST-1 stimulation in the absence of bacterial homogenate was  $69,322 \pm 8,322$  while PWM resulted in a mean of  $14,000 \pm 1,650$ .

Table 3. Co-Culture of PBMC With Lymphocyte Stimulators and Bacterial Homogenates

Co-Culture	Percent of Maximal Response	
	Non-Diseased (n = 10)	Diseased (n = 6)
TSST-1	100	100
TSST-1 + A.a.*	45 = 12	39 = 15
TSST-1 + P.g.*	29 = 17	32 = 12
PWM	100	100
PWM + A.a.*	85 = 15	95 = 12
PWM + P.g.*	105 = 18	55 = 14

Results are expressed as a mean % of the radiothymidine incorporation with either TSST-1 or PWM alone  $\pm$  standard deviation. Concentrations of TSST-1 and PWM were used which resulted in peak incorporation. Italic values indicate significant differences (Student *t*-test,  $P < 0.002$  when compared to either TSST-1 or PWM alone. No significant differences were seen when results utilizing non-diseased individuals were compared with results utilizing PBMC from individuals with periodontitis. \**A. actinomycetemcomitans* Y4, homogenate concentration used was 5 mcg/ml. <sup>†</sup>*P. gingivalis* ATCC 33,277, homogenate concentration used at 2 mcg/ml.

served for responses to PWM (Table 3). Data in Table 3 also demonstrate that immunomodulatory responses are observed in PBMC from individuals with active periodontitis. Again, the bacterial homogenates decreased the proliferative response of PBMC to TSST-1 in PBMC but had no significant effect on PWM stimulation of PBMC.

The heat lability and the influence of several endoproteases were studied to better understand the molecular make-up of the immunosuppressive material present in the bacterial homogenate. PBMC from non-diseased individuals were co-cultured with PWM and various concentrations of heat-treated *A. actinomycetemcomitans* (60°C for 15 minutes). *A. actinomycetemcomitans* Y4 ho-

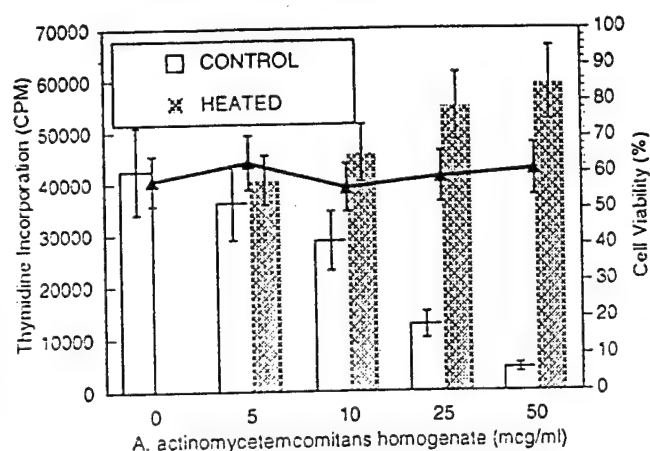


Figure 5. Effect of heat treatment of bacterial homogenate on lymphocyte proliferation and viability. PBMC were co-cultured with PWM and *A. actinomycetemcomitans* strain Y4 homogenate for 5 days. Proliferation was determined by  $^3\text{H}$ -TdR incorporation. Data represent the mean and standard deviation of four replicate cultures.

mogenate showed significantly increased lymphocyte proliferation with as little as 10 mcg/ml homogenate (Fig. 5). With regard to cell viability, heat treatment did not alter the non-toxic character of the homogenates. This is also shown in Figure 5. Endoprotease treatment of *A. actinomycetemcomitans* homogenate eliminated its immunosuppressive effect (Table 4). Viability of cultured cells remained unaltered after treatment of the homogenates with enzyme.

Superantigenic or other T-cell stimulatory activity associated with the bacterial homogenates was also analyzed. High levels of IL-2 are produced by PBMC from non-diseased patients when cultured with heat-treated *A. actinomycetemcomitans* and *P. gingivalis* homogenates (Fig. 6). Included as control are IL-2 levels produced after stimulation of PBMC with SEA, a known superantigen, and PWM, a B-cell stimulator.

The requirement for accessory cells in the induction of T-cell blast transformation was examined by monitoring  $^3\text{H}$ -TdR incorporation in PBMC. CD4<sup>+</sup> cells (helper T-cells) and CD4<sup>+</sup> cells with monocytes (Table 5) after co-culture of the cells with PWM or TSST-1, and *A. actinomycetemcomitans* Y4 antigens. CD4<sup>+</sup> cells were not responsive to PWM in the absence of B-cells and monocytes did not restore the blastogenic response of PBMC stimulated with PWM. The proliferative response of CD4<sup>+</sup> cells stimulated with TSST-1 was significantly reduced in the absence of accessory cells ( $11 \pm 7\%$  of PBMC response) but added monocytes restored the proliferative response to near normal values ( $75 \pm 15\%$  of PBMC response). The proliferative response of CD4<sup>+</sup> cells co-cultured with *A. actinomycetemcomitans* was significantly reduced ( $6 \pm 4\%$  of PBMC response) whereas monocytes restored the proliferative response to  $54 \pm 12\%$  of PBMC response.

Table 4. Influence of Proteolytic Enzymes on *A. actinomycetemcomitans* Homogenate Induced Immunosuppression

Enzyme	Percent of Maximal Response			
	TSST-1		PWM	
	Untreated	Treated	Untreated	Treated
Papain EC 3.4.22.30	20 ± 4 (60)	90 ± 15 (65)	25 ± 6 (61)	110 ± 20 (62)
Endoproteinase EC 3.4.99.30	25 ± 7 (ND)	85 ± 16 (ND)	35 ± 12 (ND)	95 ± 15 (ND)
Pepsin EC 3.4.23.1	15 ± 6 (66)	95 ± 12 (68)	38 ± 12 (64)	105 ± 17 (65)
Trypsin EC 3.4.21.4	26 ± 6 (ND)	92 ± 12 (ND)	42 ± 10 (ND)	55 ± 18 (ND)

Results are expressed as a percent of the maximal incorporation of  $^3\text{H}$ -TdR = standard deviation in the absence of bacterial homogenate for PBMC stimulated with either TSST-1 or PWM. *A. actinomycetemcomitans* Y4 homogenate was utilized at 25 mcg/ml. Values in brackets indicate the mean percent cell viability for cell populations.

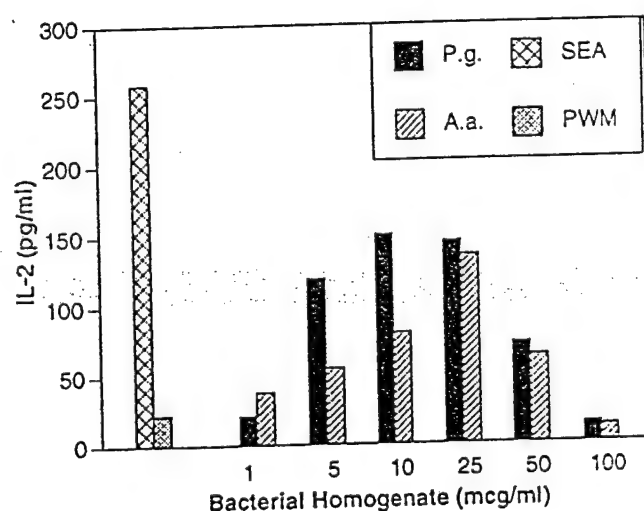


Figure 6. Influence of heat-treated bacterial homogenates on IL-2 production by PBMC cultured for 2 days. Significant production of IL-2 was observed at all homogenate doses except 1 and 100 mcg/ml (Student t-test). SEA (50 ng/ml) and PWM (25 ng/ml) were included as control.

In a third series of studies, we evaluated the influence of antibody to Class I and II major histocompatibility antigens and their influence on blocking the blast transformation response. When a heat-treated sample of *P. gingivalis* homogenate is used to stimulate PBMC from non-diseased subjects, this response is significantly reduced by adding antibody to HLA-DR into the culture system (Fig. 7). In a similar study, the addition of antibody to HLA-B failed to cause the same inhibition (data not shown).

## DISCUSSION

A consistent pattern observed was that *A. actinomycetemcomitans* and *P. gingivalis* homogenates induced lymphocyte proliferation when relatively low concentrations were employed but evoked suppression of lymphocytes at elevated levels. Significantly, PWM induced T-cell depen-

Table 5. Influence of Lymphocyte Removal From PBMC on Cell Proliferation in Response to Stimulators

Stimulator	Percent of Maximal Response		
	PBMC	CD4*	CD4 + Monocytes*
PWM (25 ng/ml)	100	5 ± 3	14 ± 8
TSST (50 ng/ml)	100	11 ± 7	74 ± 15
PHA-P (5 mcg/ml)	100	105 ± 8	54 ± 12
*A.a. (10 mcg/ml)	100	6 ± 4	54 ± 12

$10^6$  cells were cultured for 4 days in the presence of one of the stimulators. The mean incorporation of  $^3\text{H}$ -TdR by PBMC were 35,463 ± 4769; 49,234 ± 5537; 35,647 ± 2973 and 26,535 ± 2347 for PWM, TSST, PHA-P, and *A. actinomycetemcomitans* Y4\* respectively.

\*96% CD4<sup>+</sup> cells as determined by evaluation with FITC-labeled anti-CD4 staining.

\*Monocytes were obtained from homologous PBMC by adherence to collagen and were added to purified CD4<sup>+</sup> cells to make the final monocyte concentration 6%.

Italic values indicate significant differences (Student t-test,  $P < 0.05$ ) when compared to responses with CD4<sup>+</sup> cells.

dent B-cell responses were unaffected at bacterial homogenate concentrations which proved inhibitory for TSST-1 induced proliferative responses (Figs. 3 and 4). This pattern was observed using PBMC from non-diseased as well as individuals with advanced periodontitis (Table 3).

Lymphocyte blastogenic responses as seen in our studies are inversely related to the bacterial homogenate protein concentration in culture. Our results were similar to those of Shenker and co-workers<sup>2</sup> with respect to the dose-dependent inhibition at higher sonicate concentrations. Their experimental design consisted of pre-exposing *A. actinomycetemcomitans* extracts for 60 minutes to PBMC followed by the PHA, SKSD or irradiated stimulator cells. Bolton and Dyer<sup>1</sup> reported a suppressive lymphocyte response to Concanavalin A and lipopolysaccharide when exposed to purified extracellular polysaccharide from culture supernatants of *Campylobacter*. The suppression was also noted to be dose-



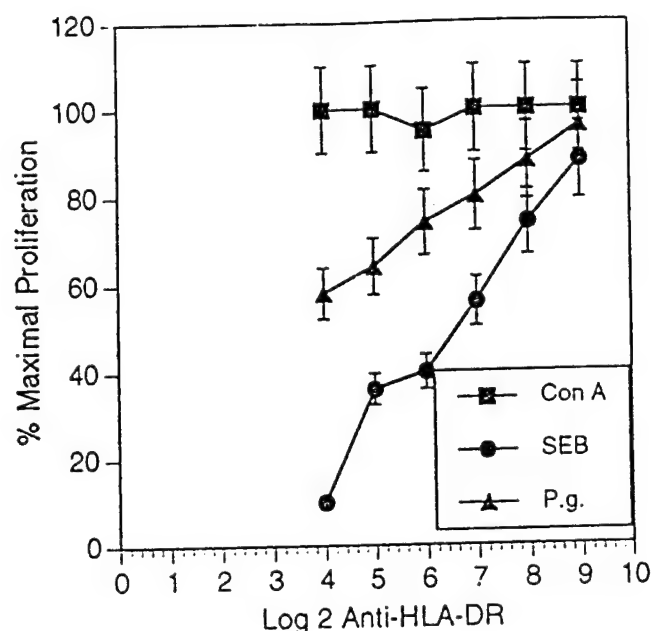


Figure 7. Influence of anti-HLA-class II antibody on proliferation of PBMC in response to Con A, SEB or *P. gingivalis* homogenate. Results are presented as the percent of maximal responses for each stimulator in the absence of antibody treatment  $\pm$  standard deviation. Effects on proliferation became significant at a Log 2 dilution of 6 for *P. gingivalis*. Homogenate induced stimulation and Log 2 dilution of 7 for SEB. No significant influence was seen when Con A was used as the cell stimulator.

dependent. In contrast, Donaldson and associates<sup>19</sup> reported proliferative and polyclonal B-cell responses at relatively high concentrations (100 mcg/ml). In these studies sonication was used to disrupt the bacteria. It is probably that, due to the generation of substantial amounts of heat during sonication, this process could affect the inhibitory factors which have been indicated to be heat-labile (Fig. 5). It appeared that the inhibitory factors decreased in activity when exposed to 60°C for 15 minutes as demonstrated by a substantial increase in the lymphocyte response at higher bacterial concentrations. This was not due to changes in toxic potential of the homogenates, since cell viability was independent of homogenate concentration whether or not the homogenate was heat treated.

Shenker et al.<sup>20</sup> characterized the lymphocyte suppressive factor of *A. actinomycetemcomitans* and indicated that it is also heat labile, trypsin and pronase sensitive, and has a molecular weight of 50,000 Daltons. The suppressor factor is likely to be a protein. We have also utilized a variety of endoprotease treatments to determine whether our suppressive activity is protein in nature. Endoprotease, pepsin, trypsin, and chymotrypsin all cause a reduction in immunosuppressive activity (Table 4).

The initial contact with virulent periodontal bacteria usually results in a heightened state of immune response. The persistence of the bacteria and their products can

eventually lead to a state of immunosuppression. There are several proposed mechanisms involving the mode of action of various suppressor factors: generation of suppressor cells, depletion of macrophages, interference with the binding of mitogens and antigens, and depletion of suppressor inducer T-cells. Seymour et al.<sup>21</sup> noted that lymphocytes extracted from periodontal tissues failed to respond to mitogens and speculated that suppressor factors may be present in vitro. Keys et al.<sup>22</sup> demonstrated that under certain experimental conditions *F. nucleatum* suppressed T-cell response but did not affect B-cell responses. Others suggested that suppressor T-cells controlled the local environment and allowed the establishment of an enriched B-cell lesion.<sup>23-25</sup> We also suggest that suppressor factors from bacteria associated with periodontitis exert differential effects on B-cells and T-cells (Figs. 3 and 4). The B-cells stimulated by PWM appear to be less sensitive to the inhibitory effects than the T-cells stimulated by TSST-1. The immunosuppressive factors may contribute to the development of the disease and the establishment of B-cells. However, there is still a need to evaluate the influence of these bacterial components on the T-cells associated with the PWM response. It is clear, however, that the periodontal status of the individual being studied does not determine whether or not a bacterial induced suppressive activity is operating. Studies utilizing PBMC from non-diseased and individuals with adult periodontitis all show the differential inhibitory effects. These results were not due to a toxic effect when cells were co-cultured in vitro with activators and bacterial products since there was no significant difference in cell viability between cultures with and without the bacterial products. Whether such differences are seen at the local (gingival tissue) level remains to be determined. Discrepancies between gingival lymphocyte and PBMC function have been reported with regard to lymphocyte subset ratio,<sup>26</sup> blastogenic response and immunoglobulin synthesis.<sup>21,27</sup>

Although periodontal lesions have been generally considered B-cell lesions. B-cells are by no means the only component of adult periodontitis.<sup>28</sup> Previous investigations did not, however, demonstrate any significant alterations in the T-lymphocyte subpopulation<sup>23,26,29</sup> of individuals with periodontitis. On the other hand, controversy still exists as to the cellular composition of the diseased gingival tissue with respect to T-lymphocyte subpopulations. T-lymphocyte subpopulations may have different phenotypic and functional properties compared to those of peripheral blood. Celenlegil et al.<sup>30</sup> found significant numbers of plasma cells throughout the connective tissues in all biopsy samples and interpreted this as a defect in the local T-cell immunoregulation.

T-cells have been shown to be involved in the regulation of polyclonal B-cell responses. Even if T-cells are not the effector element, they seem to be required for

helper influences even in mitogen and polyclonal B-cell responses. In this regard, superantigens have recently been implicated in providing a "bridge" mechanism resulting in B-cell activation in the absence of known B-cell stimulators.<sup>31,32</sup> It has been suggested that this might occur as a result of the superantigen binding simultaneously to MHC-class II determinants on the B-cell and specific TCR-V $\beta$  regions on the T-cell.

Consequently, we began a series of studies to determine if crude bacterial extracts contain superantigen-like materials. Significantly, supernatants from cultures with high proliferation rates contained elevated levels of IL-2 (Fig. 6). In addition, removal of T-cells from cultures of PBMC and semi-purified bacterial components resulted in a loss of proliferation. Of particular importance is the observation (Table 5) that T-cell response to the bacterial components was optimal only in the presence of monocytes. This would suggest the presence of superantigen since a source of MHC-class II carrying accessory cells is required for superantigen stimulated T-cell proliferation but not mitogen-stimulated responses.<sup>33-37</sup> We have also demonstrated the inhibition of *P. gingivalis* homogenate induced proliferation with antibodies to HLA Class II DR antigen (Fig. 7). Although the results are not conclusive, they suggest that superantigen-like activity can be found in *A. actinomycetemcomitans*. Direct stimulation of T-cells by antigen is probably not occurring since such induction would be monocyte independent. We are currently exploring variable expression of V $\beta$  regions in peripheral blood lymphocytes stimulated with various bacterial components.<sup>38</sup>

We have indicated that suppressive and activating factors appear simultaneously in homogenates from oral bacteria such as *A. actinomycetemcomitans* and *P. gingivalis*. By judicious use of homogenate preparation procedures and analysis of concentration effects, it is possible to study suppressive and activation influences with crude bacterial preparations. However, this would suggest that various deductions made over the years using crude preparations might need to be re-evaluated. In addition, we are currently addressing the critical need to evaluate purified preparations. A major effort is now in progress to prepare purified bacterial antigens with immunomodulatory activity. In addition, we are also separating superantigen activities from crude bacterial homogenates and evaluating them for specific stimulation of TCR-V $\beta$  regions.

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# Clinical Evaluation of Bacterial Leakage of Endodontic Temporary Filling Materials

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This study was an *in vivo* comparison of the bacterial leakage associated with three endodontic temporary restorative materials: Cavit, Intermediate Restorative Material (IRM), and TERM. The access openings of 51 endodontically treated teeth were randomly sealed with a 4-mm thickness of one of the three materials. Three wk after placement of each temporary restoration, bacterial leakage was evaluated by sampling from beneath the temporary restoration and then culturing the samples both aerobically and anaerobically. Positive growth occurred in 4 of 14 TERM samples and in 1 of 18 IRM samples. Cavit did not demonstrate leakage in any of the teeth in which it was used. Cavit provided a significantly better seal than TERM over the study period.

A major goal of endodontic therapy is to obtain and maintain a root canal system free of bacteria and other microorganisms. The purpose of a temporary restoration after initiation of endodontic therapy is to prevent the ingress of oral bacteria into the root canal system. Leakage of temporary restorations, especially those left in place for extended periods, can lead to bacterial penetration of the root canal fill (1). The ingress of microorganisms through the coronal access can complicate the course and outcome of treatment (2).

Temporary filling materials have been the subject of numerous *in vitro* evaluations that measured the ability of the materials to prevent penetration of various dyes, inks, and radiolabeled ions (3-9). Because it is the presence or absence of bacteria within the canal(s) that may determine the failure or success of endodontic treatment, such markers of leakage may not be clinically relevant because of particle sizes, which are several orders of magnitude smaller than bacteria.

Several investigators have measured the *in vitro* marginal penetration of bacteria in temporary filling materials (2, 10, 11). These studies did not reproduce the clinical environment and the functional demands to which a temporary filling is exposed. It is impossible to reproduce artificially the demands that occlusion and

the diverse nature of the oral flora place on an endodontic temporary restoration.

Studies in animals and humans attempting to evaluate the effectiveness of temporary endodontic restorative materials have flaws in their experimental design. An *in vivo* study using monkeys was flawed because the small size of the monkey teeth prohibited an adequate thickness of temporary material. The study used a 2-mm thickness of Cavit-W and concluded that this thickness was insufficient to prevent penetration of bacteria into the pulp chamber after 2 days (12). An adequate thickness of material is a prerequisite for an effective temporary restoration (6). In an *in vivo* study, eight temporary filling materials (Cavit, Cavition, gutta-percha, three types of zinc phosphate cement, and zinc oxide-eugenol) were evaluated in human patients for a minimum of 1 wk (13). Cavit and Cavition showed no or minor leakage. Gutta-percha showed gross leakage in six of eight cases, and the phosphate cements showed no leakage in more than two-thirds of the tests. Of the seven materials, only Cavit and zinc oxide-eugenol remain in common use as endodontic temporary restorative materials.

There is a need for a clinical study to evaluate the effectiveness of current and commonly used endodontic temporary restorative materials. Cavit, Intermediate Restorative Material (IRM), and TERM are commonly used endodontic temporary materials. TERM, one of the newest temporary restorative materials, is a predesigned visible, light-cured resin. Although one study has evaluated the bacterial leakage of Cavit in a clinical study, IRM and TERM have never been clinically evaluated as endodontic temporary restorative materials. The purpose of this study was to evaluate Cavit, IRM, and TERM endodontic temporary restorative materials for bacterial leakage in a human clinical study.

## MATERIALS AND METHODS

Fifty-one endodontically treated human teeth were included in the study. Teeth exhibiting large proximal, facial, or lingual restorations that might permit marginal leakage into the access chamber were excluded. The marginal integrity of all restorations was evaluated radiographically and by careful extra and intracoronary examination. Teeth with restorations of questionable marginal integrity, unopposed teeth, and teeth opposing a complete denture or distal extension partial denture were excluded. Any tooth exhibiting a fracture that might have permitted bacterial penetration through the fracture line was also excluded.

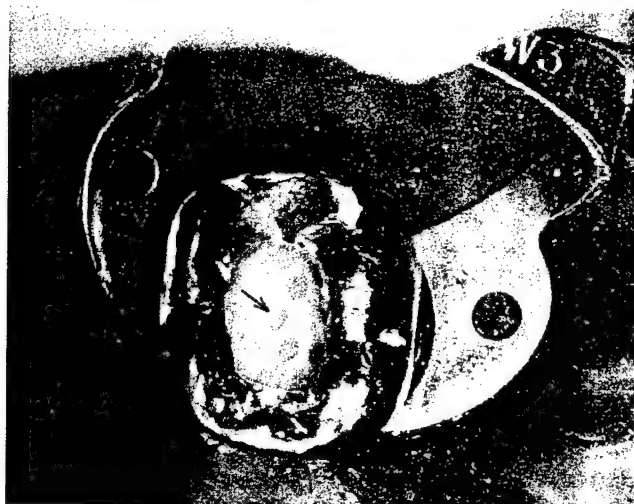


Fig 1. Paper disk on the floor of the pulp chamber. Small dots (arrow) are part of imprinted pattern on paper disks.

After rubber dam isolation and disinfection of the tooth with 5.25% NaOCl, an access was made through the coronal tooth structure, IRM temporary restoration, amalgam filling, and gold or porcelain fused to metal crown. After completion of root canal therapy, the teeth randomly received one of three temporary filling materials. These included Cavit (Premier Dental Co., Norristown, PA), IRM (L. D. Caulk Div., Dentsply Intl., Inc., Milford, DE), or TERM (L. D. Caulk). All materials were used according to the manufacturers' instructions.

#### Pulp Chamber Sampling

After obturation of the root canal system, the access chamber of each tooth was cleansed of residual root canal sealer with an alcohol-moistened cotton pellet. A sterile field was obtained by wiping the rubber dam around the tooth, the rubber dam retainer, the tooth crown, and any restoration with 5.25% NaOCl-soaked cotton pellets. The pulp chamber was then irrigated with NaOCl and a NaOCl-soaked cotton pellet placed in the chamber for 1 min (14). The pulp chamber was then irrigated with sterile saline to remove the NaOCl. Any residual saline in the pulp chamber was removed with sterile cotton pellets. Using sterile technique, pre-restoration samples for culturing and use as negative controls were taken by wiping a paper disk moistened with transport media around the pulp chamber of each tooth before placing the disk into prerduced anaerobically sterilized (PRAS) transport media. These pre-restoration controls were then plated for aerobic and anaerobic growth.

The postrestoration samples were obtained by placing a dry sterile paper disk within the pulp chamber (Fig. 1). The disk was sealed in place beneath 4 mm of temporary filling material. Filling material depth was adjusted to 4 mm by reducing the available space by inserting a combination of white stopping gutta-percha and paper disks in the bottom of the chamber. Teeth with insufficient crown height to allow a 4-mm filling depth were rejected from the study.

Patients were reappointed in 3 wk after placement of the temporary restorations to retrieve experimental samples. Teeth were again isolated with a rubber dam. The rubber dam and tooth surface were disinfected with NaOCl, and the temporary restoration re-

moved under sterile conditions. A sterile low-speed handpiece and round bur were used to remove the bulk of the material. Hand instruments were used to complete filling removal. The paper disk was removed from the chamber with sterile cotton forceps and placed in PRAS transport medium.

#### Microbiological Procedures

The PRAS transport media containing the pre- or postrestoration paper disks were diluted 1:10 with phosphate-buffered saline. Undiluted and diluted samples were plated with a spiral plater (model D, Spiral Systems, Inc., Cincinnati, OH) within 30 min of collection. Samples were incubated at 35°C under aerobic (5% CO<sub>2</sub>/air) and anaerobic conditions. Aerobic cultures were grown on Colombia agar with 5% sheep blood (CBA). Anaerobic cultures were grown on PRAS Brucella agar enriched with 5% sheep blood supplemented with vitamin K<sub>1</sub> and hemin. Aerobic growth plates were incubated for 1 wk before being discarded. The anaerobic plates were monitored for 2 wk.

Thirteen positive controls were obtained by wiping the extra-coronal surface of the experimental teeth with a sterile paper disk before isolation of the tooth surface. The paper disk was placed in transport media and cultured as described. Positive control plates were evaluated for the presence or absence of growth. Specific organisms in these plates were not identified. In addition, a large variety of known oral bacteria, both aerobic and anaerobic, were also routinely cultured to ensure that effective culturing conditions were being used.

Organisms cultured from within postrestoration samples were identified using the Minitek system (BBL Microbiology Systems, Cockeysville, MD) to determine predominant flora. Selective media and additional culturing were used as required for organism identification. Data comparisons were made using the  $\chi^2$  and Fisher's exact test.

#### RESULTS

All positive controls showed copious growth. Four of the 51 prerestoration negative controls showed positive growth. However, in no case was growth observed for postrestoration samples obtained from teeth that exhibited growth from their prerestoration control sample. Therefore, these four cases were scored as having nonleaking temporary restorations.

Of the 51 experimental samples cultured, 4 of 14 TERM samples showed positive growth, whereas 1 of 18 IRM samples and 0 of 19 Cavit samples were positive (Fig. 2). A variety of bacterial species were identified, including *Veillonella*, *Staphylococcus*, *Streptococcus*, *Peptococcus*, *Propionibacterium*, *Micrococcus*, *Hemophilus*, and *Enterobacter* species. Cavit provided a significantly better seal than TERM ( $p < 0.05$ ) over the study period. No other statistically significant comparisons were found.

#### DISCUSSION

Bacteria isolated from the positive cultures have all been implicated in endodontic infections. Therefore, their ability to penetrate endodontic temporary restorations is a cause for concern. Teeth with existing restorations that had good marginal integrity were included in the study, because unrestored teeth requiring root canal therapy are relatively rare. Therefore, making an access



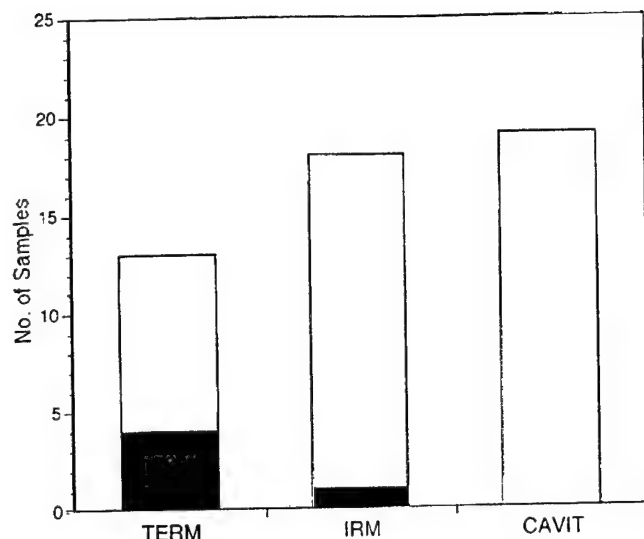


Fig 2. Experimental results. □, nonleaking; ■, leaking.

preparation through an adequate existing restoration was not only practical for this study, but it also reflects clinical practice. In this study, all but one temporary restoration was placed in a class I access preparation made through an existing restoration; one conservative class II temporary IRM restoration was placed.

Two previous investigations have evaluated the leakage of Cavit and TERM in teeth with existing restorations. In a fluid filtration study, Turner et al. (15) found that three temporary endodontic restorative materials (Cavit, TERM, and IRM) provided excellent seals for 2 wk when placed through a new amalgam restoration. In a carbon black dye study, Melton et al. (16) found that Cavit provided a more consistently effective seal than TERM and that significant leakage occurred at the permanent restoration-tooth interface. They suggested that "leaky" composite or amalgam restorations be removed, and missing tooth structure be replaced with a temporary restoration. However, the authors did not comment on the marginal integrity of the existing permanent restoration before testing. In the present study, teeth with existing restorations with questionable marginal integrity were excluded.

Positive growth found in 4 of the 50 negative controls, but not at the second sampling, can be explained in several ways. First, bacteria remaining in the pulp chamber after NaOCl disinfection and saline irrigation may have been eliminated by a possible bacteriocidal action of the temporary filling material. Second, some of the negative control growth media plates may have been contaminated, although <1% of commercially prepared plates show contamination and then only with one species of bacteria or yeast. This was shown by tracking contaminated plate records for the brand of study plates used over a 3-yr period for all experiments in the laboratory in which the experimental plates were processed. Third, bacteria that were present before placement of the temporary restoration and survived the 3-wk test period may not have been culturable upon postrestoration sample disk retrieval. Finally, it is also possible that the prerestoration control disks were contaminated somewhere between taking them out of the sterile peel pack and plating the samples in the laboratory. All four cases were scored as having nonleaking temporary restorations, because none of the postrestoration disks produced growth on plates in the laboratory.

TERM has been reported in other studies (3, 4, 7-9, 15) to provide a satisfactory seal. However, none of these studies repro-

duced the complex environment in which an endodontic temporary restoration has to function. Two in vitro studies (5, 16) found that Cavit preformed better than TERM when experimental teeth were thermocycled. In contrast, an in vitro study by Hansen and Montgomery (8) found that thermocycling did not adversely affect the seal of TERM. They found that TERM maintained an equally effective seal at 1-, 2-, 3-, and 4-mm thicknesses. They did caution, however, that TERM may be affected by occlusal loading. Quivst (17) showed that occlusal loading had a significant effect on the marginal leakage of resin restorations. TERM did not provide as effective a seal as Cavit in our study. TERM may not be able to withstand the combined clinical effects of saliva, masticatory forces, and intraoral temperature changes, as well as other materials.

A 4-mm thickness of temporary filling material was selected for this study to comply with the recommendation of Webber et al. (6). They concluded that 3.5 mm was the minimum thickness of Cavit necessary to prevent leakage. As previously mentioned, Hansen and Montgomery (8) showed that 1-, 2-, 3-, and 4-mm thicknesses of TERM provided an effective seal. TERM can be cured for 20 s with a Caulk polymerization unit (Caulk Max) in 4-mm increments (18). A 4-mm thickness of the three materials evaluated in this study was used to standardize the clinical procedures and to comply with the majority of the literature relating to temporary restoration thickness.

The present clinical study shows that Cavit provides a bacteria-free seal in conservative endodontic access cavity preparations for at least 3 wk. Although this result may be due, in part, to a bacteriocidal effect, it is significant that no bacterial colonies were grown. In addition to creating an effective seal, Cavit's use has been recommended because it is inexpensive and easy to use. There was no significant difference between Cavit and IRM. IRM is packaged as a liquid and powder that must be mixed before placement. This is a disadvantage in that the material takes considerably longer to place and adjust than Cavit.

TERM did not seem to provide a seal as effective as Cavit, and its use may be more technique-sensitive. It requires activation with a properly calibrated curing light after placement in the access cavity.

The assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Department of the Navy, Department of Defense, or the U.S. Government.

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## You Might Be Interested

A study evaluated the relation between symptoms of anxiety, as measured by Cornell Medical Index questions, and risk for coronary heart disease. Men who scored greater than 2, on a scale of 0 to 5, where 5 equaled "most anxious," had a 4.46 greater chance of sudden death from a heart attack compared to men who scored 0, i.e. no anxiety (*Circul.* 90:2225).

I guess they had something to be worried about, huh?

*Zachariah Yeomans*

## Studies of Proliferative Responses by Long-Term-Cryopreserved Peripheral Blood Mononuclear Cells to Bacterial Components Associated with Periodontitis

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Freezing techniques provide a means for repeating and extending immunological assays with frozen aliquots of an individual's peripheral blood mononuclear cell fraction. Lymphocytes which are stored frozen for a limited time retain their ability to respond to polyclonal B-cell activators, mitogens, and antigens of dental interest. Our studies extend these previous findings by determining lymphocyte functional activity following frozen storage for up to 100 weeks. In addition, the autologous immune response was measured by spontaneous lymphocyte proliferation following 0, 1, 10, 40, and 60 weeks of frozen storage. Peak responses for all individuals occurred at day 7 of incubation. The lymphocyte proliferative response to the superantigens toxic shock syndrome toxin-1 (TSST-1) and *Staphylococcus enterotoxin A* (SEA) were not changed after 100 weeks of frozen storage. Maximum responses varied among the individuals but occurred at equivalent stimulator concentrations. However, slopes generated from data obtained following 0, 4, 13, 20, 30, 50, 88, and 100 weeks of frozen storage showed no significant deviation from zero ( $P > 0.05$ ) for all individuals tested. After 100 weeks of storage, the total changes in proliferative activity (counts per minute per week) were  $-2.1\% \pm 16.8\%$  and  $-5.5\% \pm 17.0\%$  for TSST-1 and SEA, respectively. The lymphocyte proliferative responses to pokeweed mitogen, concanavalin A, and sonicates of two periodontal pathogens (*Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*) following frozen storage were similar to those with TSST-1 and SEA. These results indicate that peripheral blood mononuclear cells stored frozen may serve as appropriate controls to monitor changes in the disease state during long-term periodontal treatment.

The longitudinal evaluation of the immunological response in patients with periodontal disease, as well as a variety of other conditions, is a necessary goal that is being pursued aggressively. There has been considerable difficulty, however, because of the lack of appropriate controls. To reduce the variability inherent in many assay systems, it would be advantageous to simultaneously evaluate future samples against a standard sample. Ideally, this standard sample would be taken at the time of initial characterization or diagnosis and would be preserved for use as a reference (as treatment progresses) in future studies. One method to provide isolated cell populations as standards during treatment is cryopreservation (6).

The effects of cryopreservation on the peripheral blood mononuclear cells (PBMC) of patients with periodontal disease have not been investigated despite numerous studies evaluating the immunological status of cryopreserved cells of patients with several malignant diseases, such as leukemia (28), melanoma (10, 11, 29), breast and colon carcinomas (43), and sarcoma (31). The immunological competence of periodontitis patients is characterized by the functional activity of their immune cells (8, 27, 32, 37). Knowledge of whether these activities are intact after long-term frozen storage is critical to their use as a prognostic and diagnostic tool. Certain immunological functions have been shown to be more susceptible to cryopreservation than others even when well-established freezing and thawing techniques are used (2, 11, 19, 25, 34). Venkataraman et al. (42) showed a selective functional loss of a subset

of pokeweed mitogen (PWM)-responding suppressor T cells after cryopreservation of lymphocytes obtained from normal healthy individuals. It is necessary to clarify whether cryopreservation affects the immunological functions of cells in longitudinal studies.

A role for polyclonal B-cell activation in the pathogenesis of periodontal disease has been proposed. Significant polyclonal B-cell activation activity has been identified in sonicates of many common oral organisms (1, 33, 38). Preliminary work indicates that these periodontopathogens also carry T-cell superantigens (9). In order to set the stage for longitudinal studies involving patients with advancing periodontal disease or disease regression after initiation of therapy, we have evaluated the influence of superantigens, polyclonal B-cell activators, and extracts from periodontal pathogens on PBMC after long-term frozen storage. In addition, we also evaluated autologous immune responses in PBMC populations.

We show here that PBMC maintain functional proliferative activity after nearly 2 years of frozen storage. These positive findings now allow for the initiation of longitudinal studies aimed at accurately evaluating immunological changes in periodontitis patients as their disease is treated.

### MATERIALS AND METHODS

Cell isolation. PBMC were obtained from fresh heparinized whole blood (25 U of preservative-free heparin [Abbott Laboratories] per ml) from healthy adults. Whole blood was mixed at 2:1 with Hanks balanced salt solution, and PBMC were separated by standard Ficoll-Hypaque density centrifugation in 50-ml conical plastic tubes. Separated PBMC were washed twice in RPMI 1640 medium containing 10% A or AB+ human serum. Viable cells were transferred directly to the standard culture medium. Cells to be frozen prior to culture were transferred directly to chilled freezing medium.

Cryopreservation. Freshly washed PBMC were resuspended in freezing medium (50% pooled fetal calf serum, 10% dimethyl sulfoxide, and 40% RPMI

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TABLE 1. Relationship of cell viability to extent of time in liquid nitrogen storage

Stimulator	No. of viable cells/culture ( $10^5$ ) <sup>a</sup>				
	Fresh	Frozen 4 wk	Frozen 20 wk	Frozen 50 wk	Frozen 100 wk
None (control)	2.10 $\pm$ 0.15	1.92 $\pm$ 0.16	2.05 $\pm$ 0.09	2.10 $\pm$ 0.17	2.03 $\pm$ 0.10
TSST	2.36 $\pm$ 0.04	2.21 $\pm$ 0.06	2.30 $\pm$ 0.04	2.35 $\pm$ 0.05	2.25 $\pm$ 0.09
SEA	2.25 $\pm$ 0.12	2.37 $\pm$ 0.12	2.43 $\pm$ 0.17	2.22 $\pm$ 0.09	2.34 $\pm$ 0.23
PWM	2.34 $\pm$ 0.09	2.34 $\pm$ 0.14	2.41 $\pm$ 0.11	2.32 $\pm$ 0.13	2.39 $\pm$ 0.33

<sup>a</sup> Cultures were initially set up at  $2 \times 10^5$  PBMC per culture. Values (means  $\pm$  standard deviations) represent the cumulative totals from five individuals tested.

1640 medium) to give a final concentration of  $10^7$  PBMC per ml. Preliminary experiments showed excellent cell recovery with this medium (6). Cells to be frozen were aliquoted in plastic freezing vials in volumes of 2 ml and frozen at a rate of  $-1^\circ\text{C}/\text{min}$  to a temperature of  $-30^\circ\text{C}$  and then at  $-5^\circ\text{C}/\text{min}$  to a final temperature of  $-90^\circ\text{C}$  in a Cryotek liquid nitrogen programmable freezer (model 1010; Forma Scientific, Marietta, Ohio). Frozen samples were stored in the vapor phase over liquid nitrogen ( $-170^\circ\text{C}$ ).

Thawing. Frozen samples were rapidly transferred to a  $37^\circ\text{C}$  water bath and then subjected to sustained agitation. When only a trace of frozen sample remained, the cells were placed in 15-ml conical plastic tubes and transferred to an ice bath. The entire thawing procedure required no more than 4 min. The thawed cells were then diluted with chilled defrosting medium (50% fetal calf serum, 10% glucose, 40% Hanks balanced salt solution, and 0.05 M HEPES buffer [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] buffer [pH 7.3]) (6, 22). The first 2 ml of defrosting medium was added dropwise, and the contents of the tubes were gently mixed. The cells were gently pelleted by centrifugation at  $210 \times g$  for 15 min. The pelleted cells were resuspended in 10 ml of culture medium at  $10^5$  cells per ml without further cell washes.

Cell recovery and viability. Cell recovery and viability were assessed by phase-contrast microscopy and trypan blue exclusion. Concentrations of frozen cells were similar to the cell concentration used in the fresh-cell assays. At no time were frozen-cell concentrations readjusted or altered from those of the original cell suspension used in the fresh-cell assays.

Stimulators. Stimulators for the blast transformation assay included PWM, toxic shock syndrome toxin-1 (TSST-1), *Staphylococcus enterotoxin A* (SEA), and concanavalin A (ConA) (Sigma Chemical Co., St. Louis, Mo.). All were prepared from lyophilized preparations by suspension and dilution in RPMI 1640 medium to the desired concentration.

Bacterial extract preparation. *Porphyromonas gingivalis* ATCC 33.277 and *Actinobacillus actinomycetemcomitans* Y4 (23) preparations were grown in 1 to 2 liters of Wilkins-Chalgren broth (Oxoid Ltd, Basingstoke, Hampshire, England) supplemented with 0.3 mg of menadione per ml and 5 mg of hemin per ml. The bacterial cells were harvested by centrifugation at  $7,000 \times g$  for 15 min and washed twice in phosphate-buffered saline (PBS), pH 7.2. The organisms were weighed and suspended in a 1:2 mixture of glycerol and PBS to give approximately 0.1 g of cells per ml in the glycerol-PBS suspension. Ten-milliliter aliquots of the suspension were stored at  $-20^\circ\text{C}$  in screw-capped tubes.

Bacteria were thawed and washed three times in PBS. After being harvested, the cells were washed with Hanks balanced salt solution and homogenized in a bead mill (Mini-Bead Beater; Biospec Products, Bartlesville, Okla.) with 5-mm-diameter glass beads. The degree of disruption was assessed by phase-contrast microscopy. Protein concentrations were determined by the method of Bradford (Bio-Rad Laboratories, Hercules, Calif.). The extracts were frozen until they were used to stimulate PBMC in culture.

Blast transformation assay. Cells were assayed for blastogenic responses to bacterial preparations, antigens, mitogens, and superantigens in U-bottom mi-

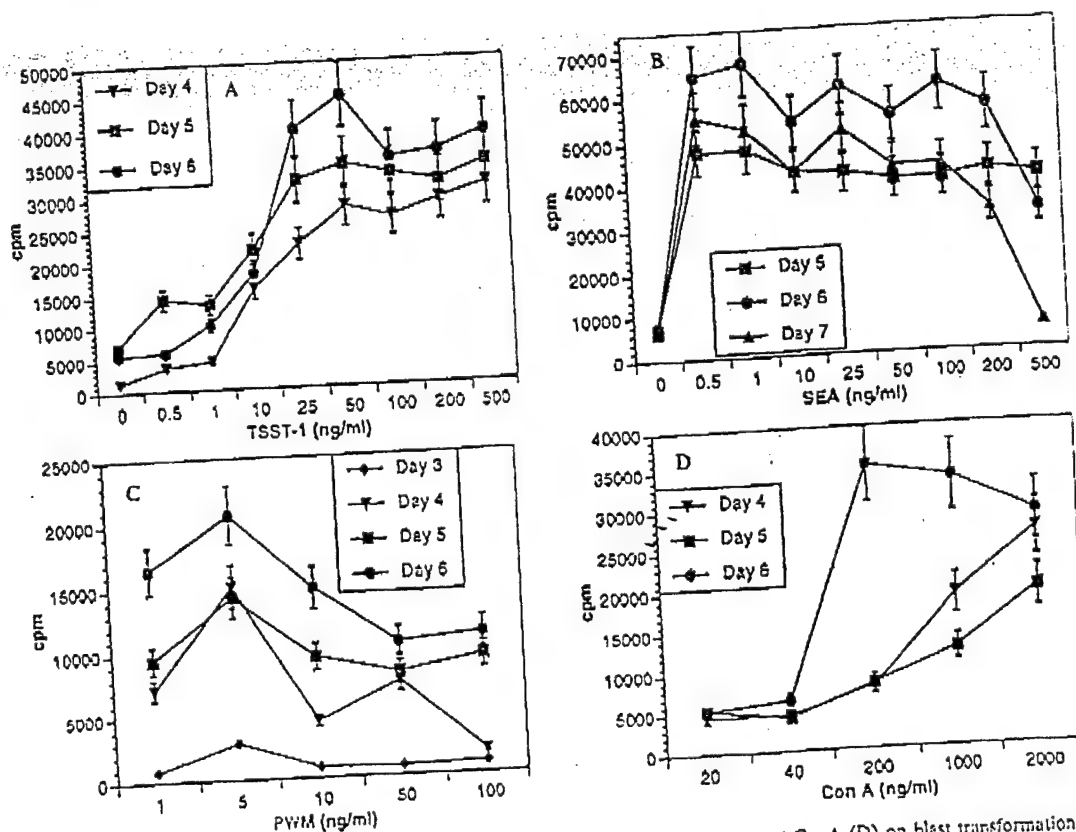


FIG. 1. Effect of concentration of and duration of incubation with TSST-1 (A), SEA (B), PWM (C), and ConA (D) on blast transformation of freshly isolated PBMC. PBMC were incubated with stimulants as indicated in and pulsed with [*methyl-3*H]thymidine 24 h prior to harvest. Data represent the median responses of quadruplicate cultures  $\pm$  standard deviations.

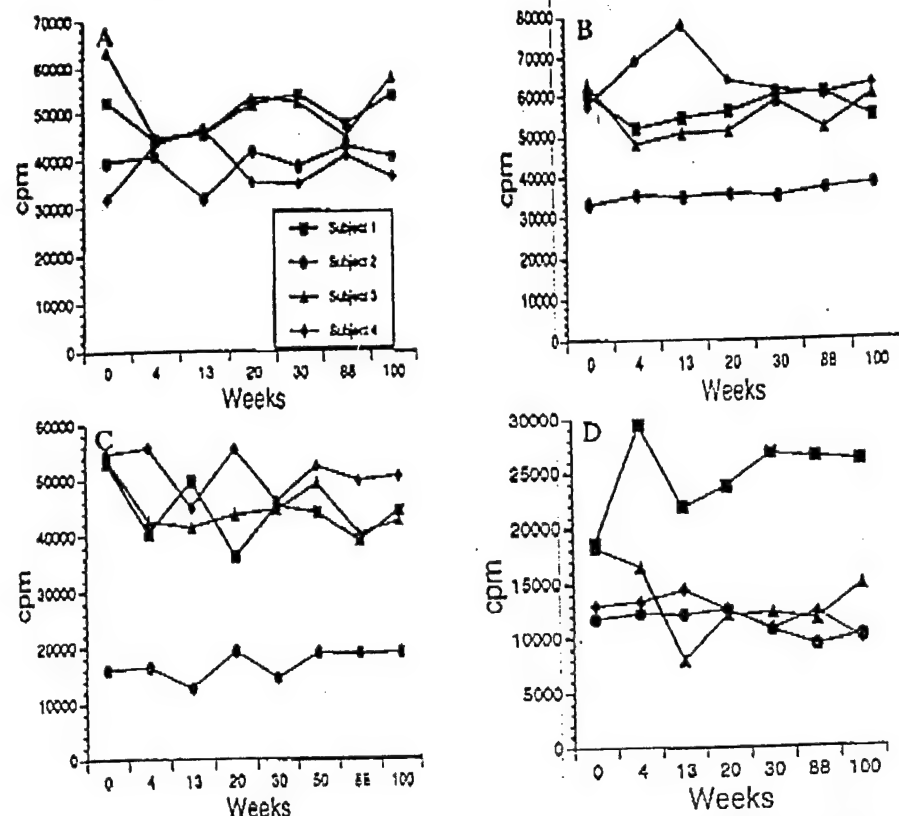


FIG. 2. Effect of TSST-1 (A), SEA (B), PWM (C), and ConA (D) on blast transformation of PBMC cryopreserved for up to 100 weeks. Thawed cells were incubated with stimulators (250 ng of TSST-1 per ml, 20 ng of SEA per ml, 5 ng of PWM per ml, and 200 ng of ConA per ml) for 6 days and pulsed with [methyl-<sup>3</sup>H]thymidine 24 h prior to harvest. The data represent the median peak responses of quadruplicate cultures at each time point. Thymidine incorporation in unstimulated cells was 5 to 5% of that in stimulated cells.

proliferation plates. Assays were done in quadruplicate wells which contained  $2 \times 10^5$  cells and stimulator in a final volume of 200  $\mu$ l.

The optimal concentration for each stimulator was determined in preliminary studies (data not shown). Prior to the initiation of the study, stimulators were aliquoted and stored at  $-70^\circ\text{C}$  to prevent degradation due to repeated freezing and thawing. Sufficient stimulator and bacterial lysate were prepared to complete the entire study, and they were thawed only one time. After 6 days of incubation in a  $37^\circ\text{C}$  humidified environment containing 5%  $\text{CO}_2$ , cells were pulsed with [methyl-<sup>3</sup>H]thymidine (1  $\mu\text{Ci}$  per well; 40 to 60 Ci/mmol). The cells were harvested 24 h later with a PHD multiple automatic sample harvester (Cambridge Technology, Inc., Watertown, Mass.). Radioactivity was determined in a Beckman LS 6500 liquid scintillation counter.

**Spontaneous proliferation.** In order to maximize the sensitivity of the spontaneous proliferation assays, studies were done during peak response times, which were between 7 and 9 days of incubation. PBMC were prepared by using the Ficoll-Hypaque procedure as described above. The cells were counted and resuspended to a final concentration of  $10^6$  cells per ml in RPMI 1640 medium supplemented with 10% human autologous serum, 1 mM L-glutamine, and 1 mM pyruvate.

Cell suspensions in 1-ml aliquots were cultured in culture tubes (12 by 75 mm) in a  $37^\circ\text{C}$  humidified environment containing 5%  $\text{CO}_2$ . [methyl-<sup>3</sup>H]thymidine (2  $\mu\text{Ci}$  per tube; 40 to 60 Ci/mmol) was added to triplicate tubes 18 h prior to harvest on days 3, 5, 7, and 9. Cells were harvested on glass discs (Schleicher & Schuell, Keene, N.H.) with a manual cell collection manifold. The filter pads were dried, and radioactivity was determined as discussed above.

**Statistical evaluations.** Slopes with standard errors and 95% confidence intervals were generated by using GraphPad Prism (GraphPad Software, Inc., San Diego, Calif.). Slopes were analyzed by using a single-sample *t* test.

## RESULTS

There were no significant changes in cell viability after 100 weeks of frozen storage. Each frozen vial was treated as if it contained the original  $2 \times 10^7$  cells. Immediately after the freezing and thawing, essentially 100% of the initial PBMC

were recovered in the thawed cultures. Therefore, there were no differences between the concentrations of the frozen cell samples and those of the original fresh samples which were cryopreserved. Even after a 6-day incubation period, there were no significant differences ( $P > 0.05$ ) between the viabilities of the fresh and frozen cultures (Table 1).

The frozen cells did not appear to undergo any changes which would alter their ability to be cultured and remain viable. The propensity for frozen cells to respond like fresh cells was demonstrated by the lack of change in the blastogenic control background counts following 24 h of incubation with [methyl-<sup>3</sup>H]thymidine (data not shown).

Blast transformation was assayed for each subject with several stimulators. Dose-response curves for each stimulator were constructed for each subject prior to freezing of the PBMC (week 0). Subsequent assays were performed under optimal incubation conditions with stimulatory doses yielding peak responses as well as suboptimal and supraoptimal doses when appropriate. In some instances, because of plateauing of the responses, studies with supraoptimal doses were not practical since very high concentrations would be required and this could influence other functional properties of the cells. Typical dose-response curves for TSST-1, SEA, PWM, and ConA are shown in Fig. 1A to D, respectively. Individual responses to TSST-1, SEA, PWM, and ConA for cells frozen for up to 100 weeks are shown in Fig. 2. The level of response for each stimulator differed among the individual subjects. Subject 2 demonstrated much lower overall responses to SEA (Fig. 2B) and PWM (Fig. 2C) than the other subjects. These individual

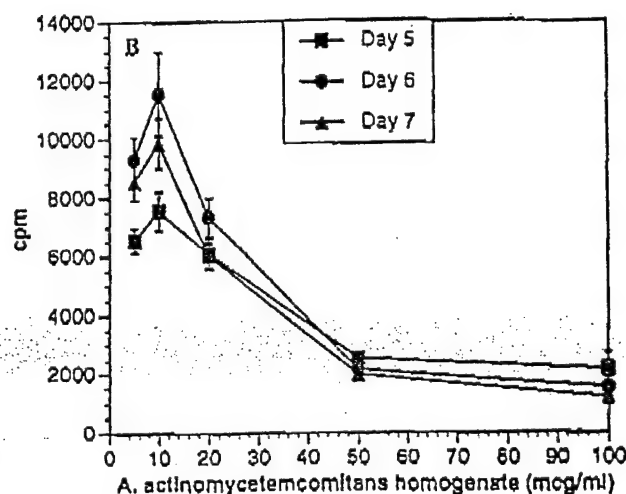
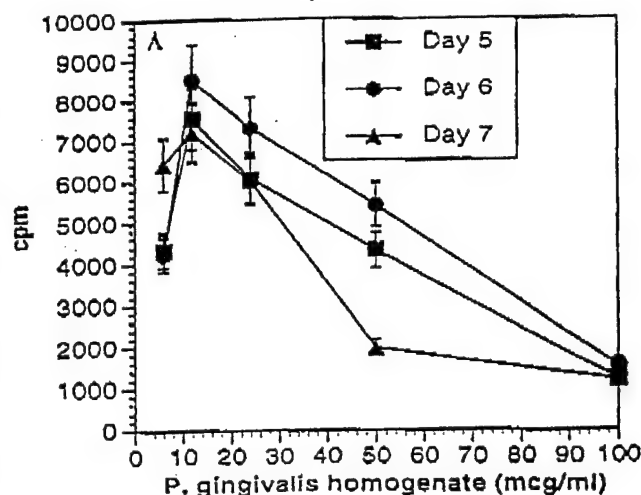


FIG. 3. Effect of concentration of and duration of incubation with *A. actinomycetemcomitans* (A) and *P. gingivalis* (B) homogenates on blast transformation of freshly isolated PBMC. PBMC were incubated with stimulators as indicated and pulsed with [ $^3\text{H}$ ]thymidine 24 h prior to harvest. The data represent the median responses ( $\pm$  standard deviations) of quadruplicate cultures.

differences in maximum response were maintained when specific indices (counts per minute for stimulated cells/counts per minute for unstimulated cells) were calculated and plotted instead of counts per minute. Although there were differences in the peak response within the subject population, each individual response was unchanged over the 100-week test period. In addition, although response levels varied, peak responses generally occurred at similar concentrations of stimulators.

Two bacterial antigen preparations were used to determine cellular blastogenic responses to periodontal pathogens. *P. gingivalis* and *A. actinomycetemcomitans* have been associated with the development of periodontitis (12, 17, 36, 44). Typical dose-response curves for homogenates of *P. gingivalis* and *A. actinomycetemcomitans* are shown in Fig. 3A and B, respectively. The effects of these components on the blastogenic response of PBMC following frozen-cell storage are shown in Fig. 4. As with TSST-1, SEA, PWM, and ConA stimulation of PBMC, the magnitudes of individual responses to the antigen preparations varied when comparisons of [ $^3\text{H}$ ]thymidine incorporation prior to freezing were made, but peak

responses occurred at similar stimulator concentrations. No changes in proliferative activity after frozen storage for 100 weeks were observed.

The maintenance of cellular proliferation following 100 weeks of frozen storage was determined by linear regression analysis. The blastogenic response of PBMC to TSST-1 in four subjects at various times after frozen storage is shown in Fig. 5. This illustrates how the data in Table 2 were generated. The 95% confidence interval for the regression line gives the range of values that defines the region containing the mean value for the relationship between time in frozen storage and PBMC blastogenic response. The overall slopes of the regression lines for all stimulators tested were not significantly different from zero ( $P > 0.05$ ). The changes in [ $^3\text{H}$ ]thymidine incorporation during peak responses were  $-2.1\% \pm 6.8\%$ ,  $-5.5\% \pm 7.0\%$ ,  $-6.7\% \pm 17.8\%$ ,  $-16.0\% \pm 37.04\%$ , and  $-9.56\% \pm 13.45\%$  following stimulation with TSST-1.

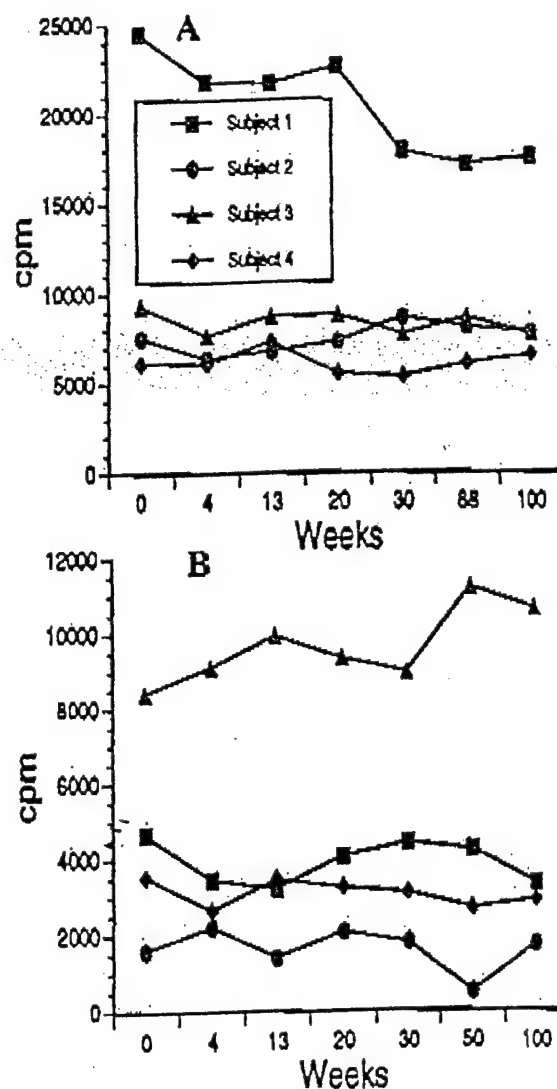


FIG. 4. Effect of *A. actinomycetemcomitans* (A) and *P. gingivalis* (B) on blast transformation of PBMC cryopreserved for up to 100 weeks. Thawed cells were incubated with 12 mg of the bacterial homogenates per ml for 6 days and pulsed with [ $^3\text{H}$ ]thymidine 24 h prior to harvest. The data represent the median peak responses of quadruplicate cultures at each time point. Thymidine incorporation in unstimulated cells was 5 to 7% of that in stimulated cells.

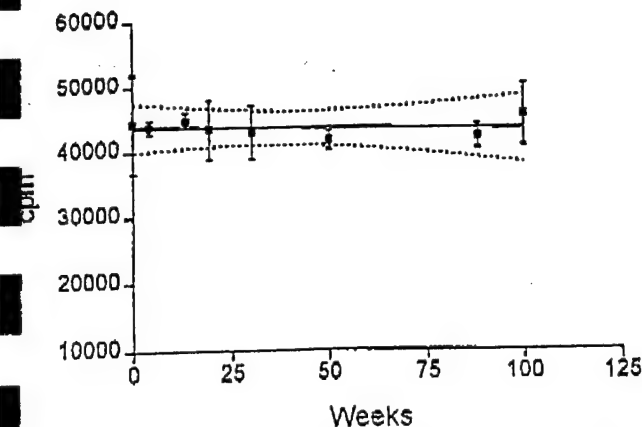


FIG. 5. Linear regression analysis of the blastogenic response of PBMC to TSST-1 stimulation at various times after frozen storage in liquid nitrogen. Cells were cultured with 250 ng of TSST-1 per ml for 6 days and pulsed with [methyl-<sup>3</sup>H]thymidine 24 h prior to harvest. Data are expressed as the means  $\pm$  standard deviations for four individual studies.

SEA, PWM, *P. gingivalis*, and *A. actinomycetemcomitans* respectively. The effects of supra- and suboptimal doses of SEA, ConA, and *A. actinomycetemcomitans* homogenates are also indicated in Table 2. Proliferative activity with TSST-1 plateaued at the lowest concentration used to stimulate the cells (Fig. 1A). Peak responses for *P. gingivalis* after 5, 6, and 7 days of incubation occurred when 12  $\mu$ g of extract per ml was added to the cultures (Fig. 3A). This and the proliferative response at a suboptimal dose are also indicated in Table 2.

Spontaneous lymphocyte proliferation (SLP) as a measure of the autologous mixed lymphocyte reaction (AMLR) was used to monitor changes in immunoregulation following frozen storage over a 60-week period. Cells were incubated for 3, 5, 7, and 9 days to determine the time needed to obtain a peak response (Fig. 6). All four subjects showed peak SLP responses at day 7. [methyl-<sup>3</sup>H]thymidine incorporation was 31,690,

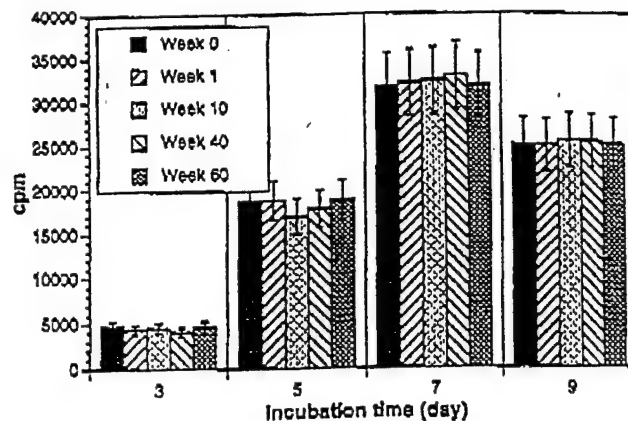


FIG. 6. SLP of cryopreserved PBMC. Data represent the medians  $\pm$  standard deviations for five individual donors for each incubation time. Assays were done in triplicate culture tubes containing  $2 \times 10^6$  PBMC in 1 ml. Cells were pulsed with [methyl-<sup>3</sup>H]thymidine 18 h prior to harvest.

32,064, 32,322, 32,903, and 31,769 cpm after 0 (control), 1, 10, 40, and 60 weeks of frozen storage, respectively, after 7 days of incubation. The effect of frozen storage on SLP of PBMC was assessed by linear regression analysis as described above. An analysis of the data (Table 3) for each incubation time (3, 4, 7, and 9 days) showed that the overall slopes of the regression lines were not significantly different from zero ( $P > 0.05$ ).

## DISCUSSION

PBMC can be maintained in frozen storage over long periods of time and subsequently show functional activity in response to a variety of stimulators, including mitogens, polyclonal B-cell activators, superantigens, and complex mixtures of bacterial extracts. The responses can be statistically evaluated so as to provide a standard control population of cells for future long-term longitudinal clinical studies.

It was not surprising that individual responses to the stimulators were varied (Fig. 2 and 4). This suggests that the recovery of activity after long-term frozen storage is not dependent on the baseline level of cell activity prior to freezing. Although the absolute responses of individuals varied, it is significant that in most cases peak responses occurred at similar stimulator dose levels. This permitted overall statistical evaluations (Table 2) to be related to specific stimulatory levels.

Since all responses were relatively constant over the course of the study period, cell populations expressing moderate activities were well maintained in culture. These cell populations could also be expected to retain other functional activities in addition to that measured by [methyl-<sup>3</sup>H]thymidine incorporation. We are currently looking at lymphokine production in

TABLE 2. Changes in the blastogenic response of PBMC to various stimulators following 100 weeks of frozen storage<sup>a</sup>

Stimulator	Concn (ng/ml)	Slope (cpm/wk)	95% Confidence interval (cpm/wk)	% Total change (mean $\pm$ SE) <sup>b</sup>
TSST	25	-14.46	-119.9 to 96.9	-0.44 $\pm$ 20.16
	120	-26.08	-111.2 to 59.0	0.51 $\pm$ 20.63
	250 <sup>c</sup>	-5.63	-18.8 to 7.50	-2.1 $\pm$ 16.8
SEA	2	-18.18	-121.0 to 84.7	-2.01 $\pm$ 16.65
	20 <sup>c</sup>	-1.71	-108.9 to 105.5	-1.68 $\pm$ 24.15
	200	-41.85	-104.9 to 21.2	-5.5 $\pm$ 17.0
PWM	5 <sup>c</sup>	-1.28	-84.7 to 82.1	-16.62 $\pm$ 36.19
	50	30.36	-62.3 to 123.0	4.15 $\pm$ 19.17
	500	-20.53	-83.8 to 42.8	-6.7 $\pm$ 17.8
ConA	20	21.01	-51.5 to 93.6	-29.15 $\pm$ 19.00
	200 <sup>c</sup>	-32.52	-60.0 to 32.0	10.18 $\pm$ 23.59
	2,000	0.76	-43.9 to 45.4	-13.54 $\pm$ 10.6
<i>P. gingivalis</i>	6,000	5.69	-19.9 to 30.4	-20.14 $\pm$ 17.42
	12,000 <sup>c</sup>	24.51	-18.8 to 67.8	-16.00 $\pm$ 37.04
<i>A. actinomycetemcomitans</i>	6,000	3.06	-28.4 to 34.5	1.48 $\pm$ 25.24
	12,000 <sup>c</sup>	8.48	-22.2 to 39.1	-9.56 $\pm$ 13.45
	25,000	0.36	-10.0 to 19.7	-11.37 $\pm$ 42.95

<sup>a</sup> Cells were stimulated for 6 days and pulsed with 1  $\mu$ Ci of [methyl-<sup>3</sup>H]thymidine 24 h prior to harvest.

<sup>b</sup> For each value,  $P > 0.05$  (not significant).

<sup>c</sup> Optimal response.

TABLE 3. Changes in spontaneous proliferation after 60 weeks of frozen storage<sup>a</sup>

Days in culture	Slope (cpm/wk)	95% Confidence interval (cpm/wk)
3	0.11	-6.58 to 6.37 <sup>b</sup>
5	8.97	-30.2 to 48.1 <sup>b</sup>
7	-10.41	-58.2 to 37.4 <sup>b</sup>
9	4.67	-22.6 to 32.0 <sup>b</sup>

<sup>a</sup> Cells were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine 24 h prior to harvest.

<sup>b</sup>  $P > 0.05$  (not significant).



order to extend our study to include a variety of functions not based on spontaneous or antigen-stimulated proliferation.

Periodontitis is generally considered a B-cell lesion. However, other immune cell components are present (3). While previous investigations have not demonstrated any significant alterations in the periodontitis T-lymphocyte subpopulation (5, 24, 40), controversy still exists as to the T-lymphocyte cellular composition of diseased gingival tissue. T-lymphocyte subpopulations in the lesion may have phenotypic and functional properties different from those of T-lymphocyte subpopulations circulating in peripheral blood. Celenligil et al. (4) found significant numbers of plasma cells throughout the connective tissues in gingival biopsy samples from subjects with rapidly progressive periodontitis and interpreted this as a defect in local T-cell immunoregulation.

T cells have been shown to be involved in the regulation of polyclonal B-cell responses. Even if T cells are not the effector element, they appear to be required for helper influences in mitogen and polyclonal B-cell responses. In this regard, superantigens have recently been implicated in providing a "bridge" mechanism resulting in B-cell activation in the absence of known B-cell stimulators (18, 41). It has been suggested that this occurs as a result of simultaneous binding of superantigen to surface major histocompatibility complex class II determinants on the B cell and to specific T-cell receptor V $\beta$  regions on the T cell.

Consequently, we have used two superantigens to stimulate PBMC cultures in an effort to determine the effects of frozen-cell storage on cell proliferation. Our data show that this response is conserved after long-term frozen storage (Fig. 2). Superantigen-induced responses involve the interaction of superantigens with T cells carrying specific T-cell V $\beta$  receptor proteins in conjunction with antigen-processing cells. We show that both of these populations of cells remain functional after the freezing and recovery procedures. Long-term frozen storage of cells responsive to superantigen stimulation is possible and should permit investigations of superantigen immunoregulation in clinical studies. Studies with control or reference cells to test simultaneously with cells recovered later in the disease process are now feasible when cellular proliferative responses are analyzed.

Under normal conditions, lymphoid cell populations do not exhibit strong proliferative reactions in culture unless provoked by an antigen or mitogen. The AMLR mediated by adult T cells is a relatively weak proliferative response that occurs in the absence of known heterologous stimuli. The AMLR represents an immunological response of T cells reactive to surface major histocompatibility complex class II antigens expressed on non-T cells which may reflect an autoregulatory immune mechanism (13, 26). The SLP response has been used to measure the AMLR of peripheral blood lymphocytes in patients with periodontal diseases (16, 35, 39). SLP is depressed in young subjects with advanced periodontitis (39), in generalized juvenile periodontitis (35), and in adult periodontitis (16). A major concern in such studies is variability in the assay method, which can be significant when appropriate controls are not used. The storage of baseline cell samples which can be reevaluated when a new sample is tested will considerably reduce this problem.

The data in Fig. 6 show that the SLP response is the most consistent function assayed in this study. In addition, there was less variation in the response among the four individuals tested compared with blastogenic transformation.

The value of being able to store peripheral blood lymphocytes in the frozen state and recover AMLR-like responses is not restricted to studies of periodontitis. Similar observations

of AMLR responses have been made in studies with Sjogren's syndrome (20, 30), Hodgkin's disease (7), acute infectious mononucleosis (21), and chronic active liver disease (45). Diseases such as gastric carcinoma (14), as well as multiple sclerosis, Guillain-Barre syndrome, acute stroke, myasthenia gravis, and seizures (15), show elevated AMLR responses.

In this study we demonstrated consistent levels of PBMC proliferative responses to various stimulatory agents, such as mitogens, polyclonal activators, and extracts of oral bacteria, after 2 years in frozen storage. We are currently investigating the stabilities of other PBMC functions, including lymphokine production, antibody production, and the ability of T cells to express cell killing, following long-term frozen storage. These results will permit better monitoring of clinical status during extensive longitudinal clinical studies.

#### ACKNOWLEDGMENT

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# Production of Interleukin-1 by Polymorphonuclear Leukocytes Resident in Periradicular Tissue

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Twenty-one patients undergoing endodontic surgery were identified. Periradicular tissue samples were recovered, and those showing significant numbers of polymorphonuclear leukocyte (PMN) infiltration were prepared for immunoperoxidase identification of interleukin (IL)-1 $\alpha$  and IL-1 $\beta$ -producing cells using specific polyclonal antibodies. In selected tissue specimens, 90% or more of the PMN's were found to stain positively for IL-1 $\alpha$  and IL-1 $\beta$ . In addition, significant numbers of plasma cells and tissue histiocytes stained positively for these IL's. Cell suspensions from selected periapical granuloma specimens, as well as from purified peripheral blood PMN's and peripheral blood mononuclear cells, were also subjected to IL-1 quantitation using a commercial ELISA procedure. Such cell suspensions were found to produce significant levels of IL and could be stimulated to produce increased levels after coculture with lipopolysaccharide. These results suggest that PMN's in inflammatory periradicular tissues may be a significant source of IL-1, and their possible roles in the establishment and resolution of periradicular lesions need to be re-evaluated.

Bacterial infection of the dental pulp and gingiva commonly results in an inflammatory cell response and subsequent destruction of alveolar bone in these areas. Periradicular pathoses can be considered multifactorial endogenous infectious diseases, with the host playing a significant role in the inflammatory process. In this regard, the chronic inflammatory cells present in large numbers in periradicular and periodontal lesions produce several highly potent cytokines, including interleukin (IL)-1 $\alpha$  and IL-1 $\beta$ , respectively, which have also been associated with bone resorption mechanisms. Dewhirst et al. (1), for example, have shown that osteoclast-activating factor is identical to IL-1. Several studies have also shown that significantly greater amounts of IL-1 can be detected in gingival fluid from inflamed sites exhibiting gingivitis than from noninflamed sites (2, 3). IL-1 $\beta$  is released in periradicular tissues

of patients with periradicular pathosis (4) and is absent in noninflamed pulpal tissues (5-7).

Although it has now been demonstrated that IL-1 may be synthesized by a number of different cell types, monocytes and macrophages are considered to be the major producers (8). In this regard, Matsuki et al. (9) have indicated that IL-1 is produced principally by macrophages in inflamed gingival tissue of humans. Recent studies, however, have shown that significant amounts of IL-1 are produced by peripheral blood-polymorphonuclear leukocytes (PB-PMN's) (10). This is of considerable interest, because PMN's, until recently, were not considered a potential source of nascent IL-1 (11). It was previously accepted that mature PMN's did not synthesize proteins (11, 12). Recent reports, however, suggest that PMN's are capable of RNA and protein synthesis, and gene production by PMN's may contribute to the inflammatory process (9).

Although the production of IL-1 $\beta$  has been extensively studied in macrophages and monocytes, it has not been fully investigated in the PMN. PMN's are the most prominent inflammatory cells in early inflammatory lesions. As the first line of defense from invasion of the host by microorganisms, they may be responsible for much of the tissue damage that occurs early in the inflammatory response. The role of the PMN needs to be re-evaluated in light of the new information suggesting that the functional activity of PMN's is more broad than previously thought.

Although several studies now form a strong case in favor of the production of IL-1 by PB-PMN's (10, 13), IL-1 has yet to be shown to be produced by PMN's resident in human inflammatory tissue or in nonexperimental inflammation. The purpose of this study was to determine whether PMN's present in human periradicular lesions produce IL-1 $\alpha$  and IL-1 $\beta$ . Utilizing immunohistochemical procedures involving specific antibodies, we have identified PMN's associated with IL-1 in fresh frozen and paraffin sections of periradicular lesions obtained from a number of subjects. Although PMN's represent a major category of cells present in endodontic and periodontic lesions, this association requires additional study to determine the ultimate role that both IL-1 and PMN's have in the establishment and development of localized oral inflammation.

## MATERIALS AND METHODS

Fresh tissue samples obtained from 21 patients undergoing endodontic root end surgery were prepared for light microscopic

studies and tissue culture. Selected patients were not currently receiving long-term anti-inflammatory medications. Each of the periradicular lesions was at least 4 mm in diameter. After excision, each of the specimens was divided into two equal sections. One section was prepared for paraffin embedding, and the second section was either quick-frozen or used in tissue culture.

Tissue fragments were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Most fresh tissue fragments were also frozen in Ames OCT compound (Miles Laboratories, Naperville, IL) in liquid N<sub>2</sub> and stored at -70°C until immunohistochemical studies were performed. For immunohistochemical analysis, 3 to 5 mm sections of either frozen or paraffin-embedded tissue were used. Frozen fragments were cut using a Reichert Histo-Stat cryostat (Warner Lambert Technologies, Inc., Buffalo, NY) at -20°C, thaw-mounted on poly-L-lysine-coated slides, and postfixed in buffered formalin for 10 min. Endogenous peroxidase activity was removed during a 20-min incubation with 0.3% hydrogen peroxide. After blocking with normal rabbit serum, the sections were treated for 3 h at room temperature with either a rabbit polyclonal antibody to IL-1 $\beta$  or IL-1 $\alpha$  (Genzyme Corp., Cambridge, MA).

Following a brief wash in phosphate-buffered saline (PBS), the sections were incubated with a biotinylated secondary antibody (Sigma Chemical Co., St. Louis, MO). With the addition of a peroxidase reagent, a stable biotin-avidin complex was formed with the bound biotinylated secondary antibody. Sites of antibody deposition were then visualized by a brown color after addition of freshly prepared substrate containing hydrogen peroxide and the electron donor chromogen diaminobenzidine (Sigma). A similar procedure was used with paraffin, except that the sections were initially deparaffinized by melting the paraffin at 55°C, soaking in xylene, and then rehydrating through graded alcohol baths. Optimum antibody dilutions were determined by staining IL-1 positive-frozen and paraffin-embedded specimens.

To determine the specificity of binding of the anti-IL-1 antibodies used in the study, dilutions of the antibodies were preincubated with either recombinant IL-1 $\beta$  or IL-1 $\alpha$  (Genzyme) at 0.5 ng/ml. Sections were also incubated with control antibodies consisting of preimmune rabbit serum and rabbit antihuman chorionic gonadotrophin. No staining occurred with any of the control antibodies (data not shown). In addition, buccal epithelial cells obtained from scrapings failed to stain with either of the anti-IL-1 antibodies. In most cases, serial sections on the same slides were used for control staining.

Human PMN or PB-mononuclear cells were isolated from blood samples collected from healthy volunteers, using sodium heparin as an anticoagulant. Blood was mixed with a solution of 6% dextran (Sigma) in 0.15 M NaCl at a ratio of 4:1 and incubated at 37°C for 1 h. PMN-rich plasma was collected and centrifuged at 275  $\times$  g for 10 min. The resultant cell pellet was resuspended in RPMI 1640 supplemented with 50% autologous plasma and centrifuged on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). Upon removal of contaminating erythrocytes by hypotonic lysis, the remaining cells were washed twice with RPMI 1640. Purity was checked morphologically using Wright-Giemsa stain (Fisher Scientific, Pittsburgh, PA), with monocyte contamination determined by nonspecific esterase staining (14). The resulting PMN's were >98% PMN and >99% viable as determined by trypan blue uptake and phase-contrast microscopy.

PBMC's were prepared using a standard Ficoll-Hypaque procedure after layering diluted blood over the Ficoll and centrifugation at 400  $\times$  g for 20 min. Cells were incubated for 24 h in RPMI

TABLE 1. No. of periradicular lesions identified with various concentrations of PMN's

Type of Lesion	Relative PMN Content (%)			
	0	1	3	5
Granuloma	7	2	3	1
Cyst	2	2	2	1
Scar	1			

0 = <5%; 1 = 5-10%; 3 = 10-20%; 5 = >20.

1640 with 10% type A human serum either with or without lipopolysaccharide (LPS).

The biopsied periradicular lesion fragments were weighed and then incubated with pronase (Sigma) at the ratio of 10 mg tissue/ml of pronase at a concentration of 1 mg/ml. After incubation at 37°C for 15 min and subsequent teasing, cells were collected by centrifugation, washed in PBS, resuspended in RPMI 1640, and incubated in triplicate at 2  $\times$  10<sup>5</sup> cells/well in 96-well microtiter plates.

To quantitate the production of IL-1 antigen, commercially available ELISA kits were used (R&D Systems, Minneapolis, MN). After pelleting of the cells, culture supernatants were collected, quick-frozen, and stored at -70°C until assayed. For quantitation of cell-associated IL-1, cell pellets were washed in PBS, resuspended in 0.5 ml PBS and frozen, and thawed three times. The supernatant was collected and tested after centrifugation at 1000  $\times$  g.

## RESULTS

After standard hematoxylin and eosin staining, tissues were evaluated for the presence of PMN's. Table 1 shows the relative PMN content and type of periradicular lesions that were available in this study. Because our interest was in evaluating the IL-1 character of PMN's, only those tissues showing significant PMN infiltration were selected for immunohistochemical staining for IL-1 $\alpha$  and IL-1 $\beta$ . In this regard, tissue from five individual donors was subjected to intensive study. It was surprising that the highest number of PMN's were found in the lesions identified as granulomas. Although the results from both the frozen sections and paraffin-embedded tissue were similar, only the paraffin-embedded material will be displayed, because its cellular morphology was superior.

The antisera used showed very good staining on sections from the diseased tissue. Many individual cells were easily identified with intense surface, as well as cytoplasmic staining. Significant numbers of PMN's, as well as other cell types (tissue histiocytes and monocytes), stained positively for IL-1 $\alpha$  and IL-1 $\beta$  (Figs. 1 to 4). It should be noted that, whereas most PMN's stained positively, there were instances where some cells in the same vicinity were positive and others were negative. In some tissue sections, portions of sections showed considerably more marked staining patterns than did others (Fig. 5). In some instances, various areas of the sections stained more dramatically than others (Fig. 6). A split-screen image is displayed in which the two areas of the same section are separated by 3 mm. The section shown in Fig. 6 was stained for IL-1 $\beta$ . Preincubation of the antisera with the appropriate recombinant IL resulted in a lack of staining (Fig. 7). In addition, in sections that remained untreated with antibody, no endogenous peroxidase staining was observed (data not shown).

To gain a quantitative understanding of the percentage of



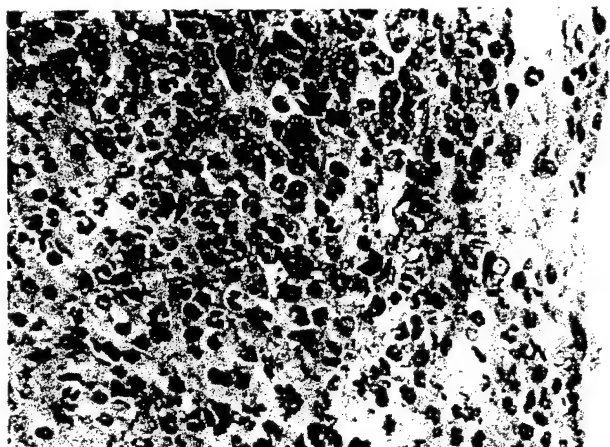


FIG 1. Survey view of a periradicular granuloma section staining positively for IL-1 $\alpha$ . Many individual cells are easily identified with an intense brown surface, as well as cytoplasmic staining. Representative cell types, including neutrophils and a plasma cell, are indicated in the figure by arrows. (Original magnification  $\times 400$ .)

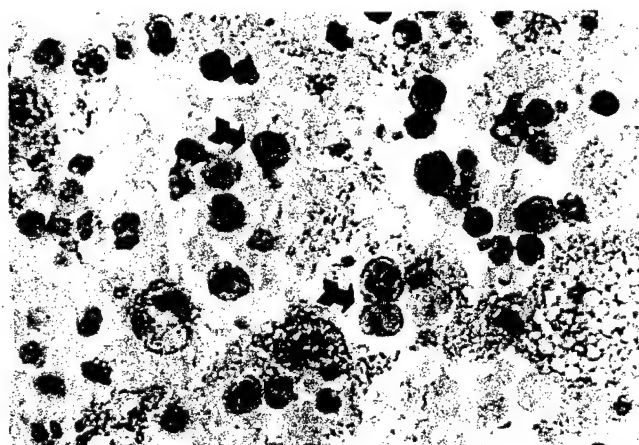


FIG 2. High magnification view of a portion of a periradicular granuloma section staining positively for IL-1 $\alpha$ . PMN's showing positive brown staining are identified by arrows. (Original magnification  $\times 1000$ .)

PMN's expressing either IL-1 $\alpha$  or IL-1 $\beta$ , 15 photomicrographs from lesion 8 were prepared and reviewed by an oral pathologist and two investigators. Identification of 100 random PMN's, plasma cells, and histiocytes was made by the oral pathologist, whereas the investigators independently determined whether the cells were stained positively for IL-1. As seen in Table 2, a significant number of the PMN's present in this surgical specimen (a granuloma) stained positively for equivalent numbers of both IL-1 $\alpha$  and IL-1 $\beta$ .

To determine whether the periradicular tissue specimens were producing IL-1 at a maximum rate and if they could be artificially stimulated to synthesize even more of these cytokines, portions of lesions 13 and 14 (granulomas representing two individual donors) were separated into individual cells using the enzyme treatment previously described. These cells, and also PMN's and mononuclear cells from peripheral blood, were then cultured for 18 h either with or without 10 mg/ml of LPS 055:B5 from *Escherichia coli*. After incubation, the amount of IL-1 $\alpha$  and IL-1 $\beta$  immunoreactivity released by the cells (Figs. 8 and 9) and that which remained cell-associated (Figs. 10 and 11) was determined using an ELISA

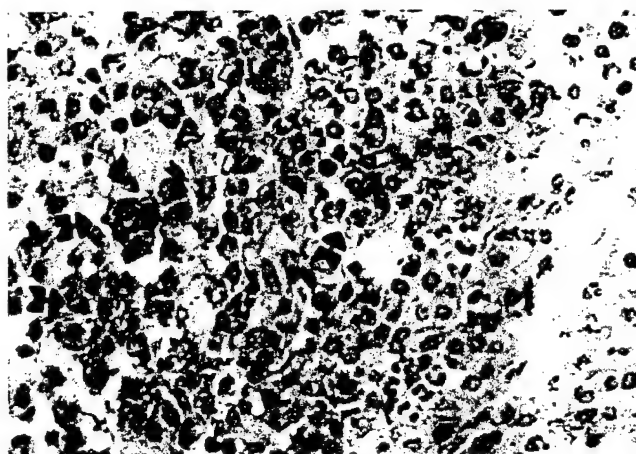


FIG 3. Survey view of a periradicular granuloma section staining positively for IL-1 $\beta$ . Note that many individual cells are easily identified with an intense surface, as well as cytoplasmic staining. Representative cell types, including neutrophils, plasma cells, and histiocytes, are indicated by arrows. (Original magnification  $\times 400$ .)

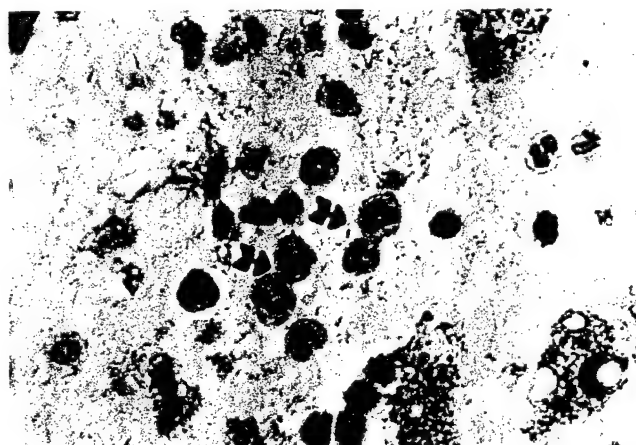


FIG 4. High magnification view of a portion of a periradicular granuloma section staining positively for IL-1 $\beta$ . PMN's showing positive staining are identified by arrows. (Original magnification  $\times 1000$ .)

procedure. IL-1 $\beta$  immunoreactivity was observed in both supernatants (Fig. 9) and lysates (Fig. 11) of the periradicular lesion material, as well as the PB-PMN's. Significantly more IL-1 $\alpha$  and IL-1 $\beta$  were seen in cultures stimulated with LPS, and more IL-1 $\beta$  was found associated with lesion cells, rather than being released into supernatants when the cells were incubated with LPS. In contrast to IL-1 $\beta$ , measurable levels of IL-1 $\alpha$  activity were detected only in cell lysates (Fig. 10), and the quantity of cell-associated IL-1 $\alpha$  measured was much less than that of IL-1 $\beta$ .

## DISCUSSION

To determine whether IL-1 is produced by PMN's, an immunohistochemical procedure was used to identify cells in periradicular lesion tissue that had associated IL-1 $\alpha$  and IL-1 $\beta$ . We have clearly shown that significant numbers of cells in the tissues were associated with such cytokines. As shown in Table 2, 92% and 93% of PMN's in the granuloma tissue were associated with IL-1 $\beta$  and IL-1 $\alpha$ , respectively. It would seem, therefore, that the number of IL-1 $\alpha$  expressing PMN's was equal to that of IL-1 $\beta$  expressing

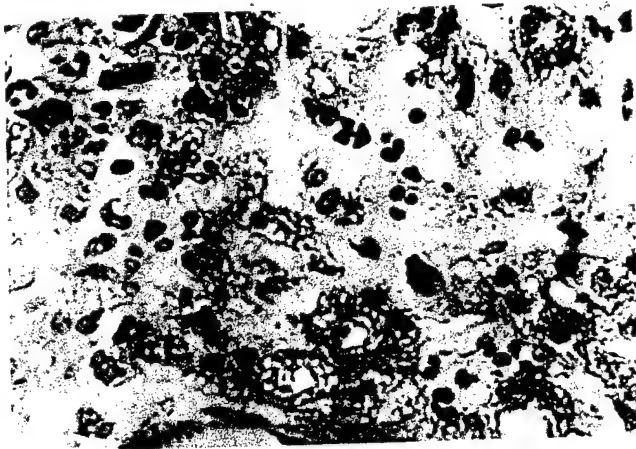


FIG 5. Survey view of a periradicular granuloma section staining positively for IL-1 $\beta$ . Note that, whereas significant staining of PMN's is generally seen, nonstaining cells are also present. One such unstained PMN is indicated by the arrow. (Original magnification  $\times 600$ .)

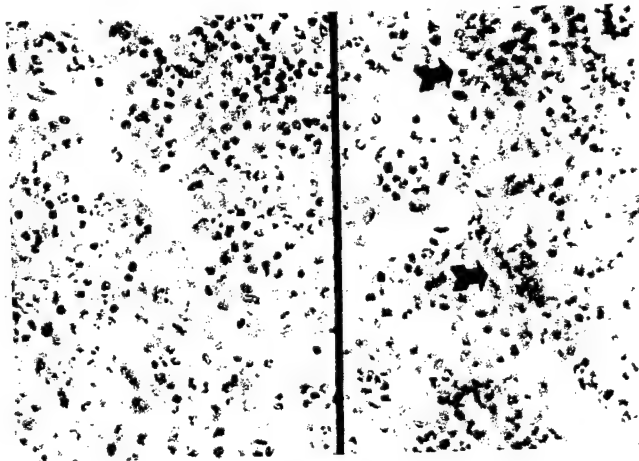


FIG 6. Split-screen image of portions of a periradicular granuloma lesion separated by 3 mm. Note that certain areas of the tissue section (indicated by arrows) stained more dramatically than others. (Original magnification  $\times 200$ .)

PMN's infiltrating the lesion, a finding similar to results for IL-1 mRNA expressing macrophages (9). In addition, significant numbers of plasma cells and tissue histiocytes were also associated with these IL's.

Our results differ somewhat from those of Artese et al. (4), who found that few cells in periapical granulomas stained positively for IL-1 $\beta$ , and those that did were monocytes. Because these investigators used a monoclonal antibody for recognizing a fragment of IL-1 $\beta$ , it is possible that IL-1 $\beta$ -producing cells were missed as a result of antigen denaturation during fixation and preparation of their tissue specimens. In addition, these investigators only tested their control antibody on fresh tissue, but utilized the antibody to identify IL-1 $\beta$  in fixed and paraffin-embedded tissue sections. In our experience, a polyclonal antibody gives much better results in identifying IL-1 $\alpha$  and IL-1 $\beta$  in formalin-fixed and paraffin-embedded tissues. Other investigators (10) have suggested that virtually all PB-PMN's show diffuse staining with both IL-1 $\alpha$  and IL-1 $\beta$  specific antisera.

It is also of interest that, in recent immunohistochemical studies involving periodontal tissue, no mention has been made of IL-1

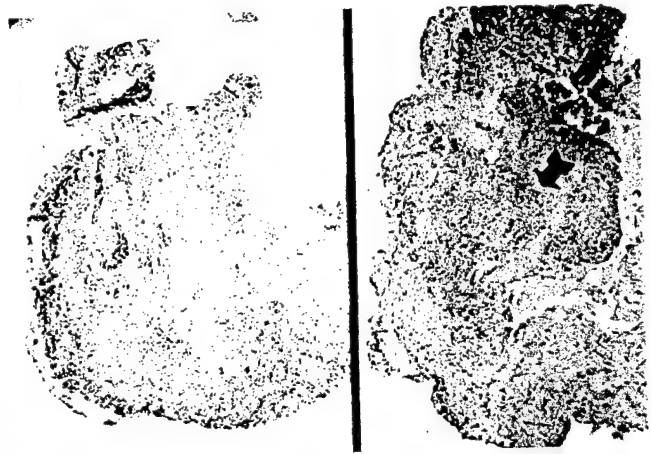


FIG 7. Survey view of tissue stained for IL-1 $\beta$ . The section on the left was treated with antibody to IL-1 $\beta$ , which had been preincubated with recombinant IL-1 $\beta$ . Note the significantly darker staining in areas of the tissue on the right (indicated by the arrow), which was stained with nonabsorbed antisera. (Original magnification  $\times 100$ .)

TABLE 2. IL-1 positive cells in lesion 8

Cell Type	Average No. of Cells Positive	
	IL-1 $\alpha$ *	IL-1 $\beta$ †
PMN	93	92 $\pm$ 7
Plasma cells	82	53 $\pm$ 6
Histiocyte	82	69 $\pm$ 15

\* Counted by a single individual.

† Counted by two individuals.

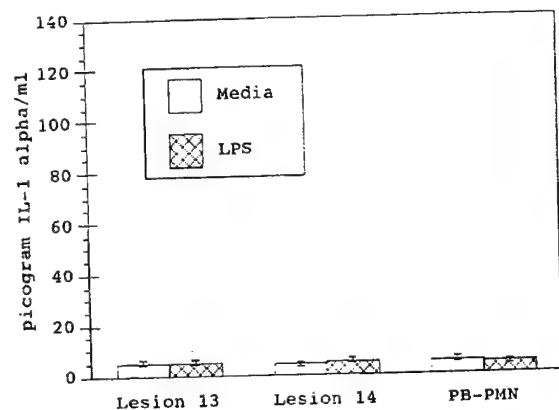


FIG 8. Production of IL-1 $\alpha$  by cells obtained from periradicular lesions and PB-PMN's. Cells were cultured in the presence or absence of 10 mg/ml of LPS, and cell culture supernatants were evaluated for IL-1 $\alpha$  using an ELISA procedure. Each value represents the mean of three individual cultures.

associated with PMN's. Jandinski et al. (15), using fluorescence staining methods, failed to identify most of the IL-1 specific cells. This could be expected, because morphological evaluations are difficult to do when using immunofluorescence methodologies. These investigators did, however, show that although individual cells in the lamina propria of gingival tissue could easily be identified, no staining was evident in the epithelium. This is consistent with our evaluation of buccal epithelial scrapings.

Although not all of the cells in a tissue section and PMN's

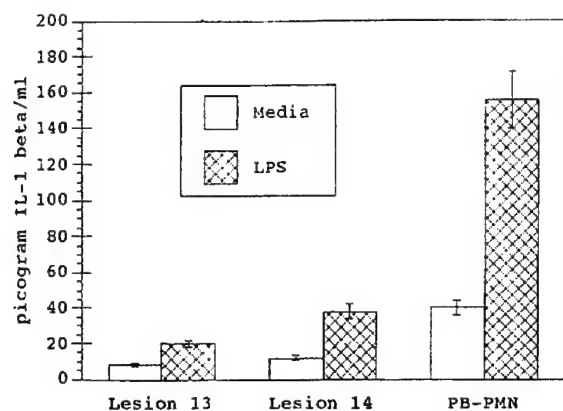


Fig 9. Production of IL-1 $\beta$  by cells obtained from periradicular lesions and PB-PMN's. Cells were cultured in the presence or absence of 10 mg/ml of LPS, and cell culture supernatants were evaluated for IL-1 $\alpha$  using an ELISA procedure. For statistical comparison, the mean  $\pm$  SE of each LPS-treated group and the unstimulated group (cultured in triplicate) were compared by Student's *t* test. LPS-stimulated IL-1 production was significantly different from the unstimulated control groups ( $p < 0.05$  for lesion 14 and  $p < 0.001$  for the PB-PMN groups).

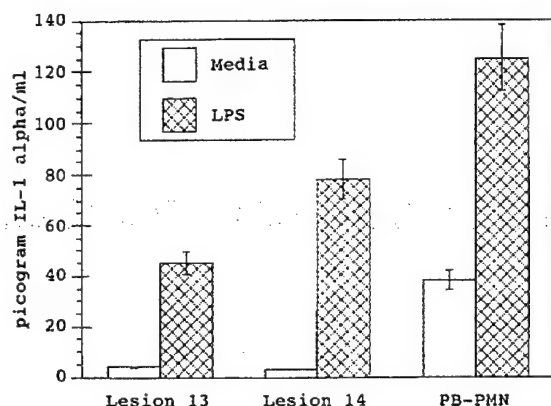


Fig 10. Production of IL-1 $\alpha$  by cells obtained from periradicular lesions and PB-PMN's. Cells were cultured in the presence or absence of 10 mg/ml of LPS, and cell lysates were evaluated for IL-1 $\alpha$  using an ELISA procedure. For statistical comparison, the mean  $\pm$  SE of each LPS-treated group and the unstimulated group (mean of three separate cultures) were compared by Student's *t* test. All LPS-stimulated IL-1 production was significantly different from the unstimulated control groups ( $p < 0.001$ ).

within a field (Figs. 1 and 3) stained positively for IL-1, the identification of PMN's producing IL-1 was not the result of either specific or nonspecific binding of IL-1 to PMN's by material synthesized by other cell types. If this were the case, IL-1-positive and -negative PMN's within the same microscopic field of view would not have been observed. The fact that IL-1-positive and -negative cells were seen indicates that PMN subpopulations may exist.

The presence of large numbers of IL-1-positive cells indicates that significant levels of IL-1 are produced by cells present in periradicular lesions. To confirm this and also to give some insight into whether or not the inflammatory cells were in an active cytokine-synthesizing state, we cultured lesion cells either in the presence or absence of LPS. The results, as shown in Figs. 9 and 11, showed that whereas a significant amount of IL-1 $\beta$  was found

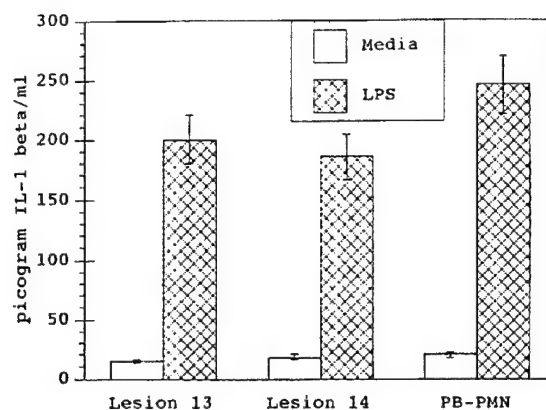


Fig 11. Production of IL-1 $\beta$  by cells obtained from periradicular lesions and PB-PMN's. Cells were cultured in the presence or absence of 10 mg/ml of LPS, and cell lysates were evaluated for IL-1 $\beta$  using an ELISA procedure. For statistical comparison, the mean  $\pm$  SE of each LPS-treated group and the unstimulated group (cultured in triplicate) were compared by Student's *t* test. All LPS-stimulated IL-1 production was significantly different from the unstimulated control groups ( $p < 0.001$ ).

in the supernatant of these cells, a significant increase was seen after incubation with LPS. As shown by other investigators, IL-1 $\beta$  is predominant in PMN's, whereas IL-1 $\alpha$  is only seen in cell lysates (10). We also observed, however, that the levels rise after stimulation with LPS. Such results indicate that the inflammatory cells within the lesions are not producing IL-1 $\alpha$  or IL-1 $\beta$  at maximum levels, because additional stimulation results in their increased production. At present it is not possible to determine which of the cells are being stimulated by the LPS to produce additional IL. The ability to stimulate PB-PMN's with LPS to produce increased levels of IL-1 is similar to that demonstrated for monocytes (16) and could suggest that Gram-negative pathogenic periodontal bacteria could also be involved in elicitation of IL-1 production by periradicular lesion PMN's. We are currently evaluating this possibility. In addition, if PMN subpopulations exist and are differentially stimulated by bacterial products, the increased IL-1 production with LPS might result from the activation of PMN's not previously activated.

IL-1 is perhaps the most widely studied cytokine in periodontal and periapical disease (2, 3, 17). The role of IL-1 is pleiotropic, in that it possesses a wide spectrum of metabolic, catabolic, immunological, physiological, and hematopoietic activities. One important role is its ability to stimulate osteoclastic bone resorption (1). Recently, Wang and Stashenko (7) determined that most bone-resorbing activity present in chronic human periradicular lesions was attributable to the action of the bone resorptive-inducing cytokines IL-1 and tumor necrosis factor (TNF), and may be responsible for reparative bone formation in the face of ongoing infection in the root canal system.

It is suggested that cytokine-mediated pathways, including cytokine-induced prostaglandin synthesis and cytokine/prostaglandin synergy, are of major importance in the pathogenesis of the human chronic periradicular lesion. A thorough understanding of cellular and plasma mediators of inflammation may provide the basis for future diagnostic indicators, as well as treatment for periradicular pathosis. The cell type responsible for producing IL-1 in periradicular lesions has, for the most part, been identified to be of the monocyte/macrophage lineage. To our knowledge, this study dem-

onstrated for the first time that IL-1 $\alpha$  and IL-1 $\beta$  can be produced by PMN's present in inflamed periradicular lesions.

PMN's were, until recently, thought to be important only in the efferent limb of the immune response and were regarded as terminally differentiated cells incapable of protein synthesis (11). PMN's are now known to synthesize a restricted but significant range of mRNA's and proteins, including IL-1, TNF- $\alpha$ , IL-6, IL-8, and IL-1 receptor antagonist (18). The most significant immunoregulatory role for PMN's has been the demonstration of IL-1 production. IL-1 has been shown to be transcribed in PMN's within 2 to 6 h of stimulation with LPS (10, 11, 19).

During the periradicular inflammatory process, IL-1 produced by PMN's may serve numerous functions: stimulation of the synthesis and release of acute phase reactants, augmentation of T- and B-cell activation, and the induction of other regulatory cytokines, such as IL-6, IL-8, and granulocyte-macrophage colony stimulating factor (GM-CSF) (8). In addition, IL-1 production by PMN's may have important autocrine and paracrine effects, including the stimulation of further IL-1 production (10, 19) and the local induction of vascular endothelial cell adhesion molecules, thereby promoting further PMN, as well as monocyte and lymphocyte, accumulation. Recent studies have also suggested that induction of IL-1 production in the PMN can be differentially regulated by various stimuli leading to a markedly different time course of production.

Marucha et al. (19) demonstrated that human PB-PMN's are able to produce IL-1 $\beta$  in response to endogenous factors, such as IL-1 $\beta$ , IL-1 $\alpha$ , and TNF- $\alpha$ . It is, therefore, important to identify the mediators and the interactions that control expression in vivo by characterizing the factors that control IL-1 $\beta$  expression in vitro. Whereas *E. coli* LPS or the nonoral organism *Fusobacterium montiferum* stimulates IL-1 production by PB-PMN's, none of a group of oral bacteria—including *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Bacteroides forsythus*—induce IL-1 production. Such organisms, however, were shown to enhance IL-1 activity and to stimulate PMN's to produce an IL-1 inhibitor (20). We are currently working to identify the nature of the IL-1 stimulators in periradicular lesions and to evaluate regulatory functions of PMN's. We are confident that this approach will lead to a better understanding of the role of the PMN in periradicular inflammation.

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We also wish to acknowledge the assistance of Teresa Gonzales, DDS, for providing expert help in the identification of cell types.

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Production of Interleukin-6 by Polymorphonuclear Leukocytes in Gingival and Periradicular Tissues. G.A. Miller, M.M.D'Alesandro and G.Euler  
Naval Dental School, Bethesda MD 20889.

IL-6 levels in polymorphonuclear leukocytes (PMN) in inflamed gingival lesions were studied. Using specific polyclonal antibody, 15-20% of the PMNs associated with tissue lesions were positive for IL-6 antigen. IL-6 positive plasma cells were also abundant. Peripheral blood PMNs (normal controls) were stimulated for 24 hrs with LPS and antigens (*Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*). Significant levels of IL-6 were produced with *E. coli* LPS B5 (933 pg/mL per 10<sup>7</sup> cells) and *E. coli* LPS B12 (791 pg/mL/10<sup>7</sup> cells) stimulation when compared with unstimulated cells (29.21 pg/mL/10<sup>7</sup> cells). Responses to bacterial extracts were dose dependent. *In situ* hybridization with cocktails containing equimolar ratios of exon/region specific probes identified IL-6 mRNA in the PMNs in the inflamed tissue lesion. The percentage of PMNs expressing IL-6 mRNA is higher than those cells expressing IL-6 antigen. Therefore, PMNs may be an additional source of IL-6 in gingival lesions. Supported by NMRDC 00095.006.0512.

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# PRODUCTION OF IL-6 BY POLYMORPHONUCLEAR LEUKOCYTES PRESENT IN PERIRADICULAR AND GINGIVAL TISSUE

G. A. Miller<sup>1,2</sup>, M.M. D'Alessandro<sup>1</sup> and G. Euler<sup>1</sup> (Naval Dental School<sup>1</sup>, Bethesda, MD and Geo-Centers, Inc.<sup>2</sup>, Fort Washington, MD)



## ABSTRACT

IL-6 levels in polymorphonuclear leukocytes (PMN) in inflamed gingival lesions were studied. Using specific polyclonal antibody, 15-20% of the PMNs associated with tissue lesions were positive for IL-6 antigen. IL-6 positive plasma cells were also abundant. Peripheral blood PMNs (normal controls) were stimulated for 24 hrs with LPS and antigens (*Actinomyces actinomycetemcomitans* and *Porphyromonas gingivalis*). Significant levels of IL-6 were produced with *E. coli* LPS B5 (933 pg/mL per 10<sup>6</sup> cells) and *E. coli* LPS B12 (791 pg/mL/10<sup>6</sup> cells). Responses to bacterial extracts were dose dependent. IL-6 production was increased 7.7-fold and 18.3-fold when cells were stimulated for 24 hr with 6 and 12 ng/mL of *A. actinomycetemcomitans* extract, respectively. *P. gingivalis* increased IL-6 production only 7-fold at the highest bacterial extract level. PMNs may be an additional source of IL-6 in gingival lesions. Supported by NMRDC 00095.006.0512.

## BACKGROUND

Two important categories of mediators of gingival and periradicular inflammation are polymorphonuclear leukocytes (PMN) and interleukins. In the pathogenesis of periodontal lesions, interleukin-6 (IL-6) has been shown to be involved by possibly stimulating osteoclastic resorption. However, IL-6 has also been shown to participate synergistically with IL-1 and to also extend the time interval until PMNs become apoptotic. The production of IL-6 has been associated with a variety of cell types including the PMN. The PMN is a terminally-differentiated cell associated with phagocytosis and bactericidal cell killing. However, the PMN also contributes to immunoregulation of both the humoral and cellular immune response. In previous studies we have shown that PMNs resident in periradicular lesions produce considerable amounts of IL-1. The purpose of this study was to determine if PMNs resident in periradicular lesions also produce IL-6.

## METHODS

Tissue fragments of periodontal granulation tissue and endodontic periradicular lesions were fixed in 10% buffered formalin and embedded in paraffin. Representative sections were stained with hematoxylin and eosin. For immunohistochemical analysis, 3 to 5 mm sections on poly-L-lysine coated slides were used. Endogenous peroxidase activity was removed with 0.3% hydrogen peroxide. After blocking with normal rabbit serum, the sections were treated for 3 hr at room temperature with either of two rabbit polyclonal antibody preparations specific for human recombinant IL-6. Following a brief wash in PBS, the sections were incubated with a biotinylated secondary antibody (Sigma Chemical Co.). In order to determine the specificity of binding of the anti-IL-6 antibodies used in this study, dilutions of the antibodies were preincubated with recombinant IL-6 (Genzyme) at 0.5 ng/mL. Sections were also incubated with control antibodies consisting of preimmune rabbit serum and rabbit anti-human chorionic gonadotropin. No staining occurred with any of the control antibodies. Serial sections on the same slides were used

for control staining.

Human PMN or peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples collected from healthy volunteers. Whole blood was mixed 1:1 with Hank's Balanced Salt Solution (HBSS) and PBMC were separated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. Separated PBMC were washed in RPMI-1640 medium (GIBCO) containing 10% A<sup>+</sup> or AB<sup>+</sup> human serum. PMNs were isolated from the Ficoll-Hypaque RBC/PMN pellet. Contaminating RBCs were removed by hypotonic lysis for 10 minutes at room temperature. PMNs were pelleted by centrifugation by 400 x g and resuspended in PBS without Ca<sup>++</sup> and Mg<sup>++</sup>. RBC debris was removed by density gradient centrifugation over a 50% Percoll cushion. PMNs recovered in the pellet were washed two times with PBS without Ca<sup>++</sup> and Mg<sup>++</sup> and resuspended. The resulting PMNs were >99% PMN and >99% viable as determined by trypan blue exclusion and phase contrast microscopy. Differential counts and morphologic examination were obtained from Diff-Quick-stained cytospins (Baxter).

*E. coli* lipopolysaccharides (LPS) B5 and B12 or extracts from *A. actinomycetemcomitans* and *P. gingivalis* were used to stimulate resting PMNs to produce lymphokine. PMNs (3 x 10<sup>6</sup>) were cultured with or without stimulants for 24 hr in microtiter plates. Supernatants were collected and evaluated for IL-6 antigen levels using enzyme-linked immunosorbent assay (ELISA) kits (Endogen). *A. actinomycetemcomitans* and *P. gingivalis* were anaerobically cultured and disrupted with a bead mill.

## RESULTS

Figure 1 is a photomicrograph of a typical periradicular granuloma showing various cell types staining positively for IL-6. Figure 2 shows a similar photomicrograph but of inflamed gingival tissue obtained from a subject with advanced adult periodontitis. Positively stained PMNs in each photomicrograph are identified by arrows. Figure 3 is a photomicrograph of a typical periradicular granuloma stained with antibody to IL-6 which was previously incubated with recombinant IL-6 (specificity control).

Fig. 1

Fig. 2

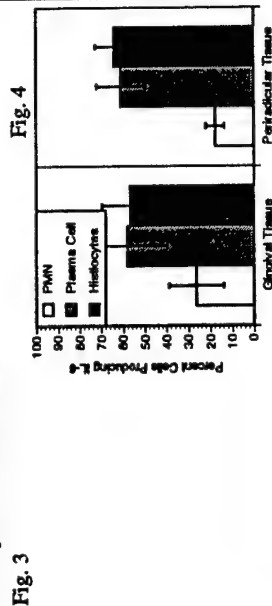


Fig. 3

In order to determine if it is possible for PMNs to be influenced by bacterial products and to produce IL-6, we cultured purified peripheral blood PMNs with two LPS preparations as well as with bacterial extracts from organisms routinely associated with periodontal lesions. These results are shown in Figures 5 and 6, respectively. The straight line shown in Figure 6 represents baseline IL-6 production in the absence of any stimulation.

Fig. 5

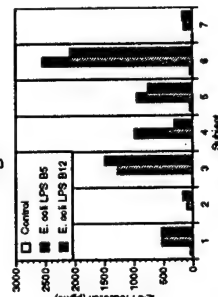
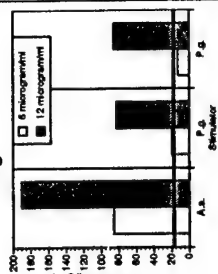


Fig. 6



## DISCUSSION

This study provides the first evidence that polymorphonuclear leukocytes resident in inflammatory periradicular lesions are able to produce substantial quantities of IL-6. It is suggested therefore, that PMNs in inflammatory lesions may represent a previously unidentified source of IL-6 and must be re-evaluated for their role in the development and resolution of such lesions. In addition, we have also demonstrated that it is possible to stimulate peripheral blood PMNs to produce IL-6 after coculture with either bacterial LPS or extracts recovered from *A. actinomycetemcomitans* or *P. gingivalis*, two oral bacteria associated with the development of periodontal lesions.

Random microscopic fields were evaluated to determine the percentage of IL-6 positive PMNs in the tissue sections. IL-6 positive plasma cells and histiocytes were similarly quantitated. (Figure 4)

Of considerable interest is the observation that, within a microscopic



# ANTIBACTERIAL EFFECTIVENESS OF TEMPORARY ENDODONTIC FILLING MATERIALS

G. Cummings<sup>1</sup>, K. Lenoci, N. Malik, M.M. D'Alesandro<sup>1</sup> and G.A. Miller<sup>1,2</sup> (Naval Dental School<sup>1</sup>, Bethesda, MD and Geo-Centers, Inc.<sup>2</sup>, Fort Washington, MD)



## ABSTRACT

The antibacterial activity of temporary endodontic filling material against several cariogenic and other oral bacteria was tested using a drop plate diffusion assay method. The antibacterial capability of Calasept, a calcium hydroxide paste (Scania Dental AB, Kivista, Sweden), Vitapex, a calcium hydroxide/iodoform paste (Neo Dental Chemical Products Co., LTD., Japan) and 40% iodoform (as found in Vitapex) were measured. Controls included sterile distilled water (negative control), an antibacterial/antifungal agent (positive control), and dental material solvents including glutaraldehyde, formaldehyde, and ethylene glycol. Zones of bacterial growth inhibition were measured in millimeters (mm) using a dial caliper. For aerobic organisms, Vitapex showed no antibacterial activity in the presence of any of the microbes tested (6.6 ± 0.4 mm). Calasept was slightly inhibitory for *E. coli* (9.1 ± 1.4 mm) and *S. aureus* (11.2 ± 1.6 mm); it was a much more effective antibacterial agent against *S. sanguis* (16.5 ± 4.2 mm) and *S. mutans* (19.67 ± 6.82 mm). Formaldehyde and glutaraldehyde inhibited bacterial growth of *S. mutans*, *S. sanguis*, *S. aureus* and *E. coli* in a dose-dependent manner. For anaerobic organisms tested, Calasept was inhibitory for *A. viscosus* (6.95 ± 1.28 mm) and *P. intermedium* (8.03 ± 1.48 mm). It was not effective against *P. gingivalis* and *V. parvula*. A 40% iodoform suspension had no effect on the growth of *A. viscosus* and *P. intermedium*. However, it inhibited growth of *P. gingivalis* and *V. parvula* on the entire plate and was bacteriocidal. Due to the varied antibacterial activities associated with Vitapex and Calasept, they are currently being evaluated for their bacteriocidal and bacteriostatic effects on several additional anaerobic microorganisms associated with endodontic infections.

Supported by Work Unit M00095.006.0511.

## BACKGROUND

The advantages of using temporary root canal filling material have been well documented for over 50 years. The criteria for an ideal temporary filling material include biocompatibility, re-sorbability, easy application and removal, radiopacity, and a broad spectrum antibacterial effect against microorganisms commonly

found in endodontic infections. The purpose of this study was to evaluate two commonly used materials in terms of their antibacterial properties against a variety of oral bacteria, particularly the anaerobes most often found in the periapical infections of dental pulps.

### Microorganisms:

Aerobic	Anaerobic
<i>Staphylococcus aureus</i> (Gram (+) cocci)	<i>Prevotella intermedium</i> (Gram (-) bacilli)
<i>Escherichia coli</i> (Gram (-) bacilli)	<i>Porphyromonas gingivalis</i> (Gram (-) bacilli)
<i>Streptococcus mutans</i> (Gram (+) cocci)	<i>Actinomyces viscosus</i> (Gram (+) bacilli)
<i>Streptococcus sanguis</i> (Gram (+) cocci)	<i>Vibronella parvula</i> (Gram (-) cocci)

## METHODS

Antibacterial activity was determined using a modified drop plate assay. Aerobic organisms were plated as an agar overlay. Anaerobic organisms, cultured in a Coy chamber, were evenly spread on the plate using a sterile swab. Wells of 2.5 to 4.5 mm diameter were made with a sterile pipet. There were no more than 5 wells per plate. Wells were filled to capacity with the material being tested and the plates incubated for 24-48 hours at 37°C. Zones of inhibition were measured using a dial caliper. The zones of inhibition included the well diameter at the widest part of the zone.

## RESULTS

Table 1. Antibacterial Activity of Dental Materials on Growth of Aerobic Microorganisms. (Activity as indicated by zones of inhibition in mm.)

Microorganism	Control	Calasept	Vitapex
<i>S. aureus</i>	6.6 ± 0.7 (n=11)	11.2 ± 1.6 (n=4)	6.6 ± 0.4 (n=4)
<i>E. coli</i>	6.7 ± 0.5 (n=16)	9.1 ± 1.4 (n=7)	7.0 ± 0.9 (n=3)
<i>S. mutans</i>	6.7 ± 0.2 (n=8)	19.7 ± 6.8 (n=7)	6.6 ± 0.3 (n=3)

Table 2. Antibacterial Activity of Dental Materials on Anaerobic Microorganisms Commonly Found in Endodontic Infections. (Activity as indicated by zones of inhibition in mm.)

Microorganism	Calasept	40% Iodoform
<i>P. intermedium</i>	8.03 ± 1.48 (n=8)	No Inhibition
<i>P. gingivalis</i>	No Inhibition	> 40 mm
<i>A. viscosus</i>	6.95 ± 11.28 (n=14)	No Inhibition
<i>V. parvula</i>	No Inhibition	> 40 mm

## DISCUSSION

Although mechanical instrumentation and irrigation greatly reduce the number of bacteria in the root canal, treated canals still harbor significant numbers of bacteria. Furthermore, these canals can become reinfected by anaerobic bacteria in periapical infections. As a result, treatment of the root canal with an antibacterial temporary filling material between appointments remains an important adjunct in the elimination of bacteria during endodontic treatment. A comparison of the antibacterial effects of these two temporary endodontic filling materials revealed significant differences in the bacteriocidal properties against different strains of bacteria. Calasept, a calcium hydroxide paste, was more effective against aerobic organisms which may penetrate the root canal system during debridement or along the margin of an inadequately sealed temporary filling. Calasept paste was also more effective against *P. intermedium* and *A. viscosus*, two anaerobic organisms commonly found in periapical infections. Vitapex, a calcium hydroxide/iodoform paste, was found to be ineffective against the selected aerobic organisms, but 40% iodoform found in Vitapex was more effective than Calasept against the anaerobes *P. gingivalis* and *V. parvula*. It can be concluded that, in certain clinical cases where one temporary paste is found to be ineffective, it would be advantageous to change to the other. It is possible that the predominant bacteria in a resistant infection would be susceptible to the second filling material.

## Risk assessment policy for evaluating reproductive system toxicants and the impact of responses on sensitive populations

G. Bruce Briggs

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### Abstract

Risk assessment policy for evaluating environmental chemicals for their potential to produce reproductive system failures is similar to policy for evaluating cancer-causing effects. The objective of reproductive system risk assessment is to expand on the test standards that primarily focus on fertility endpoints and birth defects by using mechanism-of-action studies and quantitative risk assessment methods. An understanding of the sensitivity of reproductive system insult between animal species and from animal models to man is critical to developing risk assessment policy and test standards. The reproductive process is complex and involves a number of maturation and sex cell development processes. Sensitivity to insult varies throughout this process, especially during, (1) the development of the conceptus, sperm and ova, (2) fertilization, (3) implantation, and (4) puberty. Reproductive failure has many causes and clinical effects. Risk assessment policy is directed toward reducing the uncertainty associated with the cause by providing a guide to understanding how dose, duration, and characteristics of the reproductive toxicant affect the reproductive process.

**Keywords:** Developmental toxicology; EPA risk assessment policy; Extrapolating toxicity data; Reproductive toxicology; Reproductive system risk assessment; Sperm staging

### 1. Introduction

Claude Bernard established the basic principles for toxicologic evaluation more than 100 years ago. They remain as valid today as when they were first written and are the foundation for safety evaluation research and risk assessment. This presentation will examine several of those principles and relate them to the policies that are shaping the current regulations which guide the risk assessment of potential reproductive toxicants.

Three of these principles are key factors in risk assessment policy development:

- (1) Physiologists must discover the laws of "vital manifestations" or physiological functions, and observation and examination are the only methods of investigation.
- (2) Toxicity to target organs is determined by establishing approaches to defining the mechanism of action of drugs and other chemicals.
- (3) Cause and effect relationships are established through developing an objective and a hypothesis, conducting the examination and controlling the variables.



This review presents the major reproductive risk assessment policies that are currently being used. It also discusses the "new generation" methodologies to improve the information relating to potential risk to sensitive populations; the gametes, the conceptus and the adult male and female.

## 2. Reproductive system risk assessment policy development

The science relating to the toxic insult of the reproductive system has been driven by the need to prevent exposures from reproductive and developmental toxicants to sexually active adults. Policies have evolved primarily through a series of guidelines and regulations that rely on laboratory animal surrogate models and standardized test standards that have been developed to link the causes and clinical effects of reproductive system failures (see references in Section I). The Food and Drug Administration (FDA) issued the original test standards in 1966. These guidelines established the requirements for regulatory approval of new drugs under development. Segment I Reproductive Effects Studies were proposed to evaluate fertility in rats. Segment II Teratology Studies were proposed to be conducted in a rodent and non-rodent animal model to evaluate birth defects and malformations in the offspring. Segment III Perinatal and Postnatal Studies were developed to evaluate potential toxicity to the young during lactation and early development. Multigeneration reproduction studies were also recommended for some pesticides that could enter the food chain of humans. A battery of neurobehavioral tests was established at a later date to evaluate potential developmental effects on the sensory organs and the central nervous system during pregnancy.

These tests have served the regulatory process and society by using animal surrogates as test models to avoid catastrophic toxic insult to the human reproductive system. They have not been revised since they were proposed and focus primarily on fertility endpoints of malformations, functional defects, growth retardation or death.

One approach for setting acceptable levels for developmental toxicity risk has been to use safety (uncertainty) factors. From a bioassay conducted at several dose levels in both a rodent and a non-rodent animal species, a supposedly safe dose for humans is determined by dividing the no-observable-adverse-effect level (NOAEL) by a safety factor. It has been suggested that a safety factor of 100 should be used when extrapolating from animal study data to establish acceptable human exposure levels (Lehman and Fitzhugh, 1954, Section VI). If the NOAEL is taken to be a safe dose for the experimental animals, a safety factor of 10 is suggested to allow for potentially higher sensitivities of humans compared to the experimental animals and another factor of 10 to allow for differences in sensitivities among individuals. For irreversible effects, such as death or malformation, an additional safety factor of 10 is suggested (Jackson, 1980, Section VI). Even though the safety factor of 100 is adequate to account for interspecies and intraspecies differences in response, this does not necessarily result in a risk-free dose because the power of the experiment may not be adequate to detect subtle toxic effects (Galor, 1989, Section VI). Although this method is not foolproof for setting acceptable levels for humans, it has provided a margin of safety and has reduced the risk for most chemical entities that have been evaluated by using these standardized tests. Most teratological studies are capable of detecting reproductive system disease incidence of 10% or more. The reason for this increase in sensitivity in humans to reproductive system toxicants is not clearly understood but is likely due to differences in metabolism and mechanism of action of the hazardous chemical. Warning labels are required for drugs and pesticides to alert physicians and sexually active humans to avoid contact with developmental toxicants, especially during pregnancy. The primary regulatory concern today is that data gaps exist for most of the chemicals in commerce, and more than 4000 reproductive or developmental toxicants for animals do not produce these effects in humans. About 50 human reproductive system toxicants have been reported to have caused developmental toxicity in humans (Schwetz and

Harris, 1993, Section I). The regulatory policy has been largely based on preventing or reducing exposure to the mother at or below safe levels during the sensitive periods for fetal development. Prevention of exposure to human toxicants remains to be the most effective principle for protecting the reproductive system from toxic insult (see references in Section IV).

Since the promulgation of the FDA guidelines, a number of position papers, guidelines or regulations have been written to establish policy for protecting humans from reproductive system risks. Several of these policies are discussed in this presentation and a list of policies is included.

### 3. Discussion

New risk assessment policy for going beyond the current test battery to explore the processes by which reproductive system failures and successes occur has been promulgated (see references in Section II). This policy, along with the development of new models for expanded end points that characterize mechanisms of sex cell maturation and function, is providing scientists and regulators better means for assessing chemical risks. The U.S. Environmental Protection Agency (EPA) has developed risk assessment guidelines which were finalized in 1991 (see references in Section I, 1991). These guidelines were based on the same criteria for cancer risk assessment which are routinely used today. These include:

- (1) Hazard identification
- (2) Dose-response assessment
- (3) Exposure assessment
- (4) Risk characterization

These guidelines define reproductive toxicity and developmental toxicity and describe and discuss the endpoints that need to be evaluated in order to prevent adverse effects to the reproductive system and process. The EPA guidelines define reproductive toxicity as the occurrence of adverse effects on the reproductive system that may result from exposure to environmental

agents. Toxicity may be expressed as alterations to the reproductive organs and/or the related endocrine system. The manifestation of such toxicity may include, but not be limited to, alterations in sexual behavior, onset of puberty, fertility, gestation, parturition, lactation, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive system. Developmental toxicity is defined by the EPA as the occurrence of adverse effects on the developing organism that may result from exposure before conception, during prenatal development, or postnatally to the time of sexual maturation. Adverse developmental effects may be detected at any point in the life span of the organism. The major manifestations of developmental toxicity include death of the developing organism, structural abnormality, altered growth, and functional deficiency.

Fertility and reproductive function in both males and females are evaluated in the laboratory rat in the Segment I study. The reproductive toxicity is evaluated by dosing both sexes with at least three dose levels. Sexually mature rats are dosed through a sperm cycle and female sexually mature rats are dosed with three dose levels of the test article for 14 days prior to mating. For most chemicals, only one generation is required. The offspring are then evaluated for individual and litter effects of toxicity. The U.S. Food and Drug Administration uses multigeneration studies for food additives to evaluate chemical effects on fertility, gestation, parturition, lactation development and offspring development and reproduction. Segment II developmental toxicology studies are conducted in a rodent and a non-rodent species. Pregnant rats are exposed during the period of organogenesis during days 6-15 of gestation, and pregnant rabbits are exposed on days 6-18. The pregnant dams are terminated one day prior to delivery, and the pups are examined for viability, malformation, and growth (Manson and Kang, 1994, Section VI C). The International Harmonisation Committee Guideline, (ICH, 1994, Section I), recommends the supplementation of the standardized tests with staging techniques and mechanism of action

studies. Many of these new methods are described in the references by Heindel and Chapin. (Heindel and Chapin, 1993; Chapin and Heindel, 1993, Section VI C).

The risk assessment policy guidelines have improved the earlier test standards by stating the assumptions made in the risk assessment process and standardizing the use of qualitative and quantitative data in the hazard identification and dose-response processes. The guidelines have also helped to identify research needed for reducing uncertainties and to fill data gaps. This information is included in databases (see references in Section IV), and is used along with epidemiology facts (see references in Section III), to perform a reproductive risk assessment for potential reproductive system toxicants.

The criteria that are used for cancer risk assessment are used for the risk assessment of reproductive and developmental toxicants with the following additional assumptions:

- (1) An agent that produces an adverse reproductive effect in experimental animals will potentially pose a hazard to humans after sufficient exposure.
- (2) Reproductive effects are generally the same across species except for pregnancy outcomes.
- (3) All of the manifestations of developmental toxicity are of concern, including growth alterations, functional deficits and fetal death, in addition to structural abnormalities.
- (4) A threshold is generally assumed for the dose-response curve for reproductive effects.

Standardization of data collection has been the primary objective of the International Harmonisation Committee (IHC) guidelines that were published in 1994 (IHC, 1994, Section I). This document introduces the concept of "most probable option" which is interpreted as optimizing the test parameters to reflect sound scientific procedures. This includes determining the optimal treatment period for both male and female animal models and the conceptus so that exposure to the toxicant occurs during the most sensitive period of maturation and development. The testing requirements will include general screens to identify potential treatment-related effects and studies to characterize the nature, scope

and/or origin of the toxic effect. The screening studies will remain essentially the same as with previous guidelines. The characterization studies include optimization of test designs for kinetic and metabolism studies in pregnant/lactating animals and male fertility assessment.

The IHC recommendations for male fertility assessment includes the requirement for dosing animals prior to mating for at least one sperm maturation cycle, and performing sperm evaluation studies in addition to histological evaluation. This is accomplished by incorporating methods to evaluate sperm motility and morphology using computer assisted techniques and the staging of spermatogenesis (Russell et al., 1990, Section VI C). This process can also be used for oogenesis. The ovarian follicles are all present when the female is born. They exist as primordial follicles until they are individually stimulated by hormones to develop into primary follicles, secondary follicles, early tertiary follicles and Graffian follicles. The oogenesis process involves distinct mitosis and meiosis stages. Each of these stages can be identified by characteristic structures that demonstrate the maturation and differentiation of the ova. The ability to determine the stage that the toxic insult occurred is a primary consideration in understanding how the reproductive failure was produced. The first sensitive stage of the maturation process of the gamete dictates the expression of the reproductive failure. The reproductive process is a continuum of cell growth and function. The usual end point of a toxic insult early in the development of the gamete is death to the gamete. Since there are many millions of sperm being produced simultaneously, there must be a massive insult to a majority of the sperm. This insult is detected by a loss of motility and normal morphology. Human reproductive failure can result from only a slight reduction in the number of viable sperm, but the rat has been shown to be able to produce offspring with viable sperm counts of approximately 20% of normal. Ova can be insulted at any stage of maturation, but the most rapid development stages of meiosis when the Graffian follicle is becoming functional is usually the most sensitive period for toxic insult. The "trigger points" for cause and effect relationships can occur at any

point along the maturation process. The toxic insult to the sperm can produce infertility by interfering with the locomotion process or by causing biochemical changes that interfere with fertilization. The ova may lose viability and cause infertility and early reproductive failure in animal models and premature menopause in women.

Once the stage and cell types that are affected are identified, biomarkers can be used to help develop an understanding of the mechanism of action that produced the adverse event. These biochemical markers include hormones, enzymes, DNA adducts, biochemical substrates and metabolic pathway (cytochrome P-450) pathway changes. These changes can be detected in the plasma, or the tissues of the reproductive system (see references in Section IX). This combination of assessment of the gamete maturation process, mating behavior, fertility, pre-implantation stages of the embryo, and implantation provide data for the most probable option risk assessment.

New methods models and processes have been developed to help understand the mechanism of action in the normal and abnormal reproductive process in sensitive populations (see references in Section VIII). A number of new biomarkers are currently being used to define the reproductive process and detect alterations in function that result in reproductive failures (see references in Section V). These are driving policy development and are providing a body of information that is providing the associations between causes and effects in reproductive failure. By examining similarities and common sensitivity patterns between animal species and from animals to man, an optimum risk assessment approach can be developed for environmental toxicants that affect reproduction.

New methods for utilizing all the data from animal studies for extrapolating to man (see references in Section VI A), and incorporating statistical methods (see references in Section IX A), have contributed to our ability to assess reproductive system risk. Benchmarking, comparisons of fetal-to-adult effects (A/D Ratio), and improvements in the reference dose calculations and categorical regression procedures are all being considered for improving the value of the animal data for scaling to humans (see references

in Section VI B). Standardization of test standards and comparisons of similar endpoints are critical for making risk assessments from study to study and from chemical to chemical.

Pharmacokinetic and pharmacodynamic principles can be applied for gestational and lactational modeling using physiologically-based pharmacokinetic (PBPK) procedures (see references in Section IX B).

The ultimate goal of reproductive system risk assessment policy is to provide a practical and affordable method for reducing the risk from toxicants to an acceptable level (see references in Section VII). The challenge for regulators and scientists is to reduce the variables in examining potential risk factors and to link cause and effect parameters to reflect real world scenarios.

#### 4. Conclusion

This presentation has briefly reviewed the process of reproductive risk assessment policy development and how it relates to methods and test standards that are being used to generate and evaluate the data for regulatory decision making. The conceptus and the adult male and female that are attempting to produce offspring are the most susceptible human populations to reproductive system toxicants. The reduction in variables improves the process of extrapolating data from experimental animals to humans. Reducing exposure levels to an environmental chemical or group of chemicals during sensitive periods across the human population has been the objective of regulatory agency policies, especially since 1983. Dose, duration and characterization of the risk factor(s) combined with the time of the insult and mechanism of action for reproductive failure are the primary factors in developing policies that guide reproductive system risk assessments (see references in Section VII).

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**1482** TOXICOLOGICAL EVALUATIONS OF THE CFC  
ALTERNATIVE HFC-236EA

W. J. Brock, D. P. Kelly, J. C. Stadler, G. S. Elliott, T. W. Slone, S. M. Munley, K. S. Bentley, and G. B. Briggs<sup>1</sup>. *Haskell Laboratory, E.I. du Pont de Nemours & Co., Newark, DE and <sup>1</sup>Geo-Centers, Inc., NMRI/TD, WPAFB, OH*

Toxicological evaluations were performed to assess acute, subchronic and developmental toxicity, cardiac sensitization and genetic toxicity of HFC-236ea. The 4-hr acute lethal concentration of HFC-236ea in rats exceeded 85,000 ppm; narcosis was the predominant clinical sign seen during exposure. Cardiac sensitization in beagle dogs was noted at  $\geq 35,000$  ppm with a NOEL of 25,000 ppm. No mutagenic/genotoxic activity was found *in vitro* in *S. typhimurium* and *E. coli* mutation assays or in a human lymphocyte chromosome aberration assay at concentrations of  $<100\%$ . No micronuclei were induced in bone marrow polychromatic erythrocytes in mice exposed at 5000, 25,000 or 50,000 ppm. In 2- and 13-week inhalation studies with rats exposed at 5000, 20,000 and 50,000 ppm (6 hr/day, 5 days/wk), no evidence of body or organ weight effects, clinical pathology, or induction of hepatic peroxisomes were found. Rats exposed to 20,000 or 50,000 ppm displayed a transient, diminished acoustic startle response only during exposure; rats exposed to 5000 ppm were unaffected. Microscopically, dilatation of seminiferous tubules, without effects on germ or Sertoli cells, was observed in rats at 50,000 ppm only in the 13-week study. In a rat developmental toxicity study, maternal toxicity (diminished startle response and weight gain) was evident at 20,000 or 50,000 ppm while no evidence of fetal toxicity was found in any group.

1386

CARDIAC SENSITIZATION MODEL DEVELOPMENT  
USING BIOCHEMICAL MARKERS

G. B. Briggs E. A. Smith and CAPT (S) K. Still *Tri-Service Toxicology Consortium, Building 433, 2612 Fifth Street, Wright-Patterson AFB, OH*

Chlorofluorocarbons (CFCs) are being phased out by international regulations because of negative effects on the ozone layer. Alternate fluorochemicals have been developed to replace the CFCs. Inhalation toxicology profiles have been conducted on those alternatives that are being developed for commerce. Both the CFCs and their replacement hydrofluorocarbons sensitize the dog heart to an intravenous epinephrine challenge following a ten minute exposure to the fluorochemical. This sensitization response occurs when multiple ectopic beats or atrial fibrillation is evident on electro cardiograms. The relationship between exposure levels and electrophysiological/ mechanical parameters was explored by detecting a biomedical marker in dog hearts using magnetic resonance spectroscopy imaging and magnetic resonance spectroscopy techniques. The purpose of this research was to initiate the background data for developing a new model for cardiac sensitization.

Reviewed and Revised 31 Oct 1996

1. SPONSORING ORGANIZATION: Naval Medical Research Institute Detachment (Toxicology)
2. TITLE: Enhancement of Preclinical Toxicology Characterization Methods To Evaluate Reproductive Hazards in the Navy Workplace.
3. PROJECT FOCUS: Human Health Effects, Preclinical Screening Methods
4. BRIEF PROJECT DESCRIPTION: The Navy is actively engaged in stressing reproductive health through the Occupational Safety and Health Program. Reproductive Hazards in the Workplace: A Guide for occupational Health Professionals was issued in 1992 to provide consistent and uniform guidance to Navy occupational health professionals. An important aspect of this information is the evaluation and management of reproductive hazards in the environment. The Naval Medical Research Institute/Toxicology Detachment (NMRI/TD) is responsible for developing the biomedical data necessary to characterize the toxicity of materials of interest to the Navy and use these data to formulate occupational and environmental health-hazard evaluations and risk assessments. This includes establishing appropriate personnel exposure limits which address Navy-Specific circumstances of exposure. The pervasiveness of chemicals and chemical mixtures in the Naval environment with estrogenic activity is driving the need for a new approach to monitor and study them. A functional toxicology approach is being developed at NMRI/TD which is based on screening tests for molecular constructs containing specific receptor and reporter genes to study endocrine system activation. These studies will then guide the reproductive and developmental toxicology studies performed in laboratory animals to characterize the toxicity and predict acceptable exposure levels. This combination of mechanism of action studies and enhanced animal bioassays will provide the biomedical data to assist in assuring the readiness of Navy personnel.
5. SELECTED REFERENCES:

Reproductive Hazards in the Workplace : A Guide for Occupational Health Professionals (1992). Navy Environmental Health Center Technical Manual NEHC-TM92-2, May

Briggs, G. Bruce, (1996). Risk assessment policy for evaluating reproductive system toxicants and the impact of responses on sensitive populations. TOXICOLOGY 111 NOS. 1-3. 305-313.
6. KEYWORD FOR INVENTORY CATEGORY IN NEEDS DOCUMENT: Methods

Reviewed and Revised 31 Oct 1996

7. KEYWORD FOR INVENTORY SUBCATEGORY IN NEEDS DOCUMENT: Hazard identification, toxicology characterization, database development, exposure assessment and decision-making

8. KEYWORDS FOR EXPERIMENTAL SYSTEMS/SPECIES: IN VITRO, receptor assays, cell culture, ELISA: INVITRO, embryo culture, sperm and ova function, mammalian reproduction and developmental toxicology: INCOMPUTERO, mathematical modeling, risk assessment

9. KEYWORDS FOR EXPERIMENTAL ENDPOINTS: Reproductive, male and female hormones, sperm motility and mortality, ova development, histopathology, functional toxicology, gene expression, immunotoxicology, neurotoxicology, biochemistry, pharmacokinetics, risk factors, risk assessment

10. KEY WORDS FOR CHEMICALS UNDER STUDY: PCBs, DBNP, dioxins, heavy metals, solvents, hydraulic fluids, surety chemicals, TMPP, alkylphenols, isocyanates, free-radical producers

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Reviewed and Revised 31 Oct 1996

1. SPONSORING AGENCY: Naval Medical Research Institute Detachment (Toxicology)
2. TITLE: Neuroendocrine Pathways Controlling Male And Female Reproduction Associated With The Gulf War Syndrome.
3. PROJECT FOCUS: The hormonal regulation of the male and female gonad represents a novel method of toxicology that requires assessment of functional endpoints that measure the pituitary-adrenal-gonadal feedback mechanisms. To simply assess gonad changes histologically or through fertility endpoints provides limited data relating to mechanism of action of reproductive toxicants. The staging of the reproductive cycle in both sexes is critical to the understanding of how endocrine disruptors mimic or interrupt hormone function. Mean gonadotrophin levels, pulsatility, endocrine system sensitivity and an understanding of the normal function (control animals), is essential to the interpretation of effects of xenobiotics on the reproductive process. The monitoring and measuring of chemical biomarker endpoints that evaluate pituitary sensitivity, adrenal function and gonadal response and normal fertilization and maintenance of the conceptus. The evaluation of neuroendocrine function by evaluating potential sites of toxic vulnerability requires study of endocrine support mechanisms during gonad development, fertilization, implantation, pregnancy, birth and post partum development of the infant.
5. SELECTED REFERENCES:

Culler, Michael D. (1996). The Female Reproductive System-How to Assess Potential Toxicity. Continuing Education Course PM #11, Society of Toxicology 35th Annual Meeting, Anaheim, California, March.

Abou-Donia, M.B. et.al., (1996). Neurotoxicity resulting from coexposure to pyridostigmine bromide, DEET, and permethrine: Implications of Gulf War chemical exposures. Journal of Toxicology and Environmental Health, 48:35-56.
6. KEYWORD FOR INVENTORY CATEGORY: Methods
7. KEYWORD FOR INVENTORY SUBCATEGORY: Hazard identification, military toxicology, Gulf War Syndrome, neuroendocrine disruption or mimicking, rodent biomarkers
8. KEYWORDS FOR EXPERIMENTAL SYSTEM/SPECIES: IN VIVO, rat: INVITRO, neuroendocrine bioassays, chickens
9. KEYWORDS FOR EXPERIMENTAL ENDPOINTS: Neurological, reproductive, military toxicology, Gulf War Syndrome, exposure identification, toxicology characterization, risk assessment,

**Reviewed and Revised 31 Oct 1996**

physiologically based-pharmacokinetics (PBPK) methods

10. KEYWORDS FOR AGENTS UNDER STUDY: Pyridostigmine bromide, DEET, permethrine, pesticides, ozone depleting substances, DBNP, solvents, methoxyfluorine, pentobarbital, methoxychlor, surety chemicals

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1. SPONSORING AGENCY: Naval Medical Research Institute Detachment (Toxicology)
2. TITLE: The Evaluation Of PCBs In The Military Environment.
3. PROJECT FOCUS: Endocrine Disruption by PCBs
4. BRIEF PROJECT DESCRIPTION: Exposure in the military environment to hormonally active chemicals by humans and animals can produce a wide range of abnormal phenotypes including masculinized and defeminized females and feminized and demasculinized males. Areas to be demilitarized, remediated and civilianized may contain significant contamination from PCBs that must be removed or reduced. Disposal of surplus hardware and vessels may release PCBs into the environment. Navy environments on ships and during training and battlefield scenarios may produce low level exposure to PCBs in areas where electric capacitors and transformers are present. The predominant effects reported during human exposures to PCBs for endocrine system toxicity suggest that the primary adverse health effects attributable to high occupational PCB exposures are produced following dermal exposure. PCBs alter sex differentiation and reduce fertility by affecting breeding performance without altering sperm and testicular measurements. The primary mechanism of PCB reproductive toxicity is thought to be through hormonal activity which alters the internal endocrine environment or causes central nervous system interference with hormone release or function. The effects have multiple endpoints which create the rational approach to testing by using a combination of fertility, morphology, hormone and chemical bioassay methods to measure toxicity from environmental exposure to PCBs. A battery of tests is currently in place within the Tri-Service Toxicology Consortium to measure and monitor xenobiotics and their clinical effects. The endocrine battery of screening tests for endocrine disruption will be added to the toxicology research evaluation process, and PCBs will be used to validate the test procedures as more is known about the endocrine endpoints associated with PCB exposure than any other chemical from the military environment.
5. SELECTED REFERENCES:

Moore, J.A. et al., (1995). An evaluative process for assessing human reproductive and developmental toxicity of agents. Reproductive Toxicology, Vol 9. No.1. 61-69

Briggs. G Bruce, (1996). Risk assessment policy for evaluating reproductive system toxicants and the impact of responses on sensitive populations. TOXICOLOGY 111. 305-313
6. KEYWORD FOR INVENTORY CATEGORY: Methods

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7. KEYWORDS FOR INVENTORY SUBCATEGORY: Military toxicology, human risk assessment, endocrine disruptor testing

8. KEYWORDS FOR EXPERIMENTAL SYSTEM/SPECIES: IN VIVO mammalian rodent studies, hormone assays, biological chemicals monitoring, morphological evaluations

9. KEYWORDS FOR EXPERIMENTAL ENDPOINTS: Neurological, reproductive, hormone receptors, hormone assays, histopathology, risk assessment

10. KEYWORDS FOR AGENTS UNDER STUDY: PCBs

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1. SPONSORING AGENCY: Naval Medical Research Institute Detachment (Toxicology)
2. TITLE: The Evaluation Of DBNP For Endocrine Effects.
3. PROJECT FOCUS: Developmental toxicology with hormone disruptor assays
4. BRIEF PROJECT DESCRIPTION: 2,6-di-t-butyl-4-nitrophenol (DBNP) may be an unintentional artifact derived during operation of electrostatic precipitators responsible for removing particulate oil from the atmospheres aboard U.S. Navy submarines. The general and reproductive toxicity of DBNP is relatively unknown. Research is being conducted to characterize the toxicity of DBNP. These will include chemical characterization, target organ toxicity studies, subchronic toxicity study, reproduction study, developmental toxicity study and risk assessment. The reproductive assessment will include the International Harmonisation Committee recommendations for sperm staging and characterization and the E-Battery of tests to assess the potential for endocrine disruption. The endpoints of sperm motility and morphology, hormone assays, and fertility impairment will be evaluated and appropriate exposure levels will be established for the submarine atmospheres. The E-Battery will be collaborated with the guidelines being established for assessing endocrine disruptor potential of chemicals of military interest. The methods will be validated by using a known endocrine disruptor in the military environment. These data will be combined with the classical toxicology studies to provide the fleet with risk assessment data to assure readiness of the military personnel.
5. SELECTED REFERENCES:

Vesselinovitch, D. et al., (1961). Mammalian toxicity and histopathologic effects of 2,6-dibutyl-4-nitrophenol. Toxicol Appl Pharmacol 3 713-725

Gilbert, D., et al., (1969). Effect of substituted phenols on liver weights and liver enzymes in the rat: structure-activity relationship. Food & Cosmet Toxicol 7. 603-619
6. KEYWORD FOR INVENTORY CATEGORY: Methods
7. KEYWORDS FOR INVENTORY SUBCATEGORY: Exposure risk assessment, toxicology characterization
8. KEYWORDS FOR EXPERIMENTAL SYSTEM/SPECIES: Laboratory studies, mammalian toxicity, IN VIVO testing

Reviewed and Revised 31 Oct 1996

9. KEYWORDS FOR EXPERIMENTAL ENDPOINTS: Toxicology studies, E-Battery for endocrine disruptors, hormone receptors, sperm and ova development assessment, histopathology, biochemical assays, metabolism studies, risk assessment

10. KEYWORDS FOR CHEMICALS UNDER STUDY: DBNP, Dinitrophenols, DNP

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Reviewed and Revised 31 Oct 1996

1. SPONSORING ORGANIZATION: Naval Medical Research Institute Detachment (Toxicology)
2. TITLE: The Role Of Oxidative Stress In Producing Reproductive Toxicity By Evaluations In Surrogate Animal Models.
3. PROJECT FOCUS: Human health effects
4. BRIEF PROJECT DESCRIPTION: The Naval Medical Research Institute Detachment (Toxicology) has proposed to develop methods and data applications for the prevention and intervention of reproductive toxicity associated with oxidative stress. Free radical mediated oxidative stress has been shown to be involved in the impairment of reproductive system function in both males and females. Navy personnel must perform their mission in training and battlefield scenarios that may contain hazardous chemicals. The reproductive consequences of chronic low level exposures to environmental contaminants is largely unknown. Detection of xenobiotics in human and animal tissues cannot be used as evidence of a reproductive system intoxication. Studies on PCBs to assess sperm function in rats has suggested that enzymatic antioxidant defense mechanisms of the testes are impaired. A relation between oxidative stress mechanisms and endocrine disruption has been made, and future studies within the Tri-Service Toxicology Consortium are proposed to study the role of reactive oxygen species with hormonal levels in rats.
5. SELECTED REFERENCES:

Fantel. A.G., (1996). Reactive oxygen species in developmental toxicity: review and hypothesis. Teratology 53. 196-217

Foster, W.G. et al., (1996). An overview of some reproductive toxicology studies conducted at Health Canada
6. KEYWORD FOR INVENTORY CATEGORY FROM NEED DOCUMENT: Methods
7. KEYWORD FOR INVENTORY SUBCATEGORY FROM NEED DOCUMENT: Hazard identification, biomarkers, exposure and risk models, basic and applied research, sentinel species, database development
8. KEYWORDS FOR EXPERIMENTAL SYSTEM/SPECIES: IN VIVO, INVIVO, EX VIVO, mammalian animals, laboratory study
9. KEYWORDS FOR EXPERIMENTAL ENDPOINTS: reactive oxygen species, hormonal measurements, biomarkers, immunological, reproductive, hormone receptors, histopathology, molecular, PBPK models, risk assessment, risk factors

Reviewed and Revised 31 Oct 1996

10. KEYWORDS FOR AGENTS UNDER STUDY: PCBs, TCDD, cocaine, anticonvulsant drugs, transient heavy metals, benzene derivatives, hyperbaric environments

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# POSTER SESSION PRESENTATION ABSTRACT

Navy Occupational Health and Preventive Medicine Workshop  
DO NOT FOLD!

ASSESSMENT OF ENDOCRINE DISRUPTORS IN THE NAVAL ENVIRONMENT. G. Bruce Briggs, K. R. Still, and W. W. Jederberg. Geo-Centers, Inc ; Naval Medical Research Institute Detachment (Toxicology)

The Navy has a risk reduction program that provides occupational health professionals with guidance that stresses reproductive health. The NMRI/TD is responsible for developing the data necessary to characterize the toxicity of materials of interest to the Navy and for formulating occupational and environmental health hazard evaluations and risk assessments. A growing body of evidence has begun to suggest that chemicals in the public environment may be producing adverse health effects by disrupting endocrine system function. The Navy is correlating research with the CENR Endocrine Disruptor Work Group to determine potential health and ecological effects, risk uncertainties and research needs. Results of activities to develop methods and predictive models and measurements will be presented.

CATEGORY (Circle one): (1) Occ. Hlth. (2) Prev. Med. (3) Research/Development  
(4) Hlth. Promtn. (5) Industrial Hygiene

FORMAT (Circle one): (1) Research Poster (2) Informational Poster

SENIOR AUTHOR'S SIGNATURE GB Briggs PHONE# 937 255-6188 DATE       

USE OF CLOTHING IMPREGNANTS AND SKIN REPELLENTS FOR PERSONAL PROTECTION AGAINST MEDICAL IMPORTANT ARTHROPODS.  
LT WILLIAM H. DEES, MSC, USNR, LT MICHAEL J. BANGS, MSC, USNR  
AND LT ANTHONY KISZEWSKI, MSC, USNR. NAVY DISEASE VECTOR  
ECOLOGY AND CONTROL CENTER, ALAMEDA, CALIFORNIA

In many combat and disaster situations, the success of a mission depends on personal protection against biting arthropods.



PRINT CLEARLY AND INCLUDE ALL AUTHORS LISTED ON THE ABSTRACT

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TOXICOLOGICAL EVALUATION OF EXPOSURE TO TWO  
FORMULATIONS OF A PYROTECHNICALLY-GENERATED AEROSOL.  
ELDON A. SMITH\*, EDGAR C. KIMMEL\*, HM2 JEFF L. CASSELL,  
JAMES E. REBOULET\* AND ROBERT L. CARPENTER\*. NAVAL  
MEDICAL RESEARCH INSTITUTE DETACHMENT (TOXICOLOGY)  
AND GEO-CENTER, INC\*., WRIGHT-PATTERSON AFB, OHIO.

Fischer 344 rats (250-300 g) were exposed to the by-products of two formulations of SFE Formulation A, a pyrotechnically-generated aerosol fire extinguishant. Exposures levels ranged from 50 g/m<sup>3</sup> (nominal concentration) to 240 g/m<sup>3</sup>. The length of exposure was either 15 or 60 minutes under static conditions. A 700 L whole-body inhalation chamber was used for the exposures and consisted of a supply/exhaust system, aerosol generator and exhaust scrubber. The chamber atmosphere underwent aerosol [size (MMAD), distribution (sg) and concentration] and gas [CO, CO<sub>2</sub> and O<sub>2</sub>] analysis. Clinical observations were taken throughout the exposure. Animals were euthanized 1-hr post-exposure and underwent histo- pathological examination and blood gas analysis. The difference in formulation caused dramatic results in survivability. The first formulation produced levels of carbon monoxide very close to lethal concentrations, while the second formulation showed little if any production. The lack of carbon monoxide production during pyrolyzation is the key difference in the survivability and toxicity of the two formulations.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF SFE FIRE  
SUPPRESSANT ATMOSPHERES IN SMALL VS LARGE SCALE TESTS:  
IMPLICATIONS FOR PULMONARY DEPOSITION AND TOXICITY.  
EDGAR C. KIMMEL\*, ELDON A. SMITH\*, JAMES E. REBOULET\* AND  
ROBERT L. CARPENTER. NAVAL MEDICAL RESEARCH INSTITUTE  
DETACHMENT (TOXICOLOGY) AND GEO-CENTERS, INC.\*,  
WRIGHT-PATTERSON AFB, OH.

A comparison of the physical and chemical properties of SFE (a dry powder) fire extinguishant atmospheres was made between those generated to assess inhalation toxicity in a whole-body exposure system and those generated in facilities used to evaluate fire extinguishment efficacy. The purpose was to establish a basis for extrapolation of laboratory toxicity testing data to field conditions. Nominal concentrations of 50 and 80 g/m<sup>3</sup> in both systems were examined. Mass concentration and concentration decay of the aerosol component, aerosol size distribution and particle growth, and concentration profile of CO and CO<sub>2</sub>, the major gaseous components of SFE atmospheres, were determined. At comparable nominal concentrations, the initial mass concentration of SFE aerosols in the large system was 1.5 times greater than that in the exposure chamber. Aerosol concentration in both systems decayed exponentially. Hygroscopic growth of the particles was greater and more rapid in the large system. Gas concentrations in the larger test system also were greater by a factor of 1.5. However, in this system gas concentrations also underwent exponential decay whereas gas concentrations in the exposure chambers were steady state over the 1 hour test periods. Laboratory exposures at the higher target load concentrations produced pulmonary edema and elevated HbCO in rats. Theoretical, mathematical expressions (humans) characterizing CO<sub>2</sub> ventilatory effects on particle deposition and for HbCO formation were derived as a basis for comparison of the potential toxicity of the atmospheres.

EVALUATION OF BRONCHOALVEOLAR LAVAGE  
FROM RATS EXPOSED TO A PYROTECHNICALLY  
-GENERATED AEROSOL. K S Zepp, E A Smith, S L  
Prues, E C Kimmel, J E Reboulet and R L Carpenter.  
Tri-Service Toxicology Consortium, Building 433, 2612  
Fifth Street, Wright-Patterson AFB, OH.

Fischer 344 rats (250-300 g) and Hartley guinea pigs (300-400 g) were exposed to the by-products of SFE, a pyrotechnically-generated aerosol fire extinguishant. Exposures levels ranged from 50 g/m<sup>3</sup> (nominal concentration) to 240 g/m<sup>3</sup>. The length of exposure was either 15 or 60 minutes. A 700 L whole-body inhalation chamber was used for the exposures and consisted of a supply/exhaust system, aerosol generator and exhaust scrubber. Exposures were conducted under static chamber conditions. The chamber atmosphere underwent aerosol [size (MMAD), distribution ( $\sigma_g$ ) and concentration] and gas [CO, CO<sub>2</sub> and O<sub>2</sub>] analysis. Animals were euthanized one hour post-exposure. Half of the animals underwent bronchoalveolar lavage (BAL) and half underwent histopathological examination. The BAL was analyzed for total protein, acid and alkaline phosphatase,  $\beta$ -glucuronidase, lactate dehydrogenase and cell viability. An increase in several of the enzymes were noted as the concentration and length of exposure increased.

CROSSMATCHING BLOOD TYPES FROM FOUR  
RAT STRAINS. HMC S M Bulger and E A Smith.  
Tri-Service Toxicology Consortium, Building 433,  
2612 Fifth Street, Wright-Patterson AFB, OH.

The ability to collect multiple blood samples from a single animal without disrupting the pharmacodynamics of the animal or endangering its life is of concern when conducting pharmacokinetic or metabolism studies. However, if an equal amount of blood could be transfused to the animal after each collection, it would reduce the likelihood of altered pharmacodynamics or endangerment to life. The objective of this research was to determine if blood from one animal could be transfused to another and if there are incompatibilities between strains. The study examined four rat strains (Fischer, Sprague-Dawley, Wistar and Long Evans). Each strain consisted of four males. Four milliliters of whole blood were collected from each animal for crossmatching. Blood samples were first crossmatched with each animal within its strain and then with each animal of the other three strains. The blood samples were then pooled by strain and the pooled samples crossmatched against each other. Finally, all four strains were pooled together and crossmatched. No incompatibilities were found within each of the strains or between them, whether individual or pooled.

THE USE OF PUSH/PULL CANNULATION AS A VIABLE ALTERNATIVE IN MULTIPLE BLOOD SAMPLING. S L Prues, E A Smith, HMC S M Bulger, K S Zepp and HM2 J L Cassell. Tri-Service Toxicology Consortium, Building 433, 2612 Fifth Street, Wright-Patterson AFB, OH.

A method was developed for collecting multiple blood samples without disrupting the pharmacodynamic of the subject. The study consisted of implanting cannulas in the femoral artery and vein of four Fischer rats. Three-way stop-cocks were placed at the end of each cannula. Animals were placed in nose-only holders. A 1 mL whole blood sample was collected from the arterial cannula at the following time points: 0, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 minutes. Following each collection time point, an equal volume of whole blood (1 mL) was infused back into the animal via the venous cannula. The donor blood was collected from animals of the same sex, strain and age and crossmatched and pooled in a satellite blood collection system containing Citrate-phosphate-dextrose-adenine (CPDA-I). Blood samples were evaluated for pH, blood gases ( $pO_2$  and  $pCO_2$ ), bicarbonate, hemoglobin levels, glucose, electrolytes, total protein, lactate dehydrogenase (LDH), creatine kinase (CPK), and blood urea nitrogen (BUN).

TOXICOLOGICAL EVALUATION OF EXPOSURE TO  
TWO FORMULATIONS OF A PYROTECHNICALLY  
-GENERATED AEROSOL. E A Smith, E C Kimmel,  
HM2 J L Cassell, J E Reboulet and R L Carpenter.  
Tri-Service Toxicology Consortium, Building 433, 2612  
Fifth Street, Wright-Patterson AFB, OH.

Fischer 344 rats (250-300 g) were exposed to the by-products of two formulations of SFE Formulation A, a pyrotechnically-generated aerosol fire extinguishant. Exposures levels ranged from 50 g/m<sup>3</sup> (nominal concentration) to 240 g/m<sup>3</sup>. The length of exposure was either 15 or 60 minutes under static conditions. A 700 L whole-body inhalation chamber was used for the exposures and consisted of a supply/exhaust system, aerosol generator and exhaust scrubber. The chamber atmosphere underwent aerosol [size (MMAD), distribution ( $\sigma_g$ ) and concentration] and gas [CO, CO<sub>2</sub> and O<sub>2</sub>] analysis. Clinical observations were taken throughout the exposure. Animals were euthanized 1-hr post-exposure and underwent histopathological examination and blood gas analysis. The difference in formulation caused dramatic results in survivability. The first formulation produced levels of carbon monoxide very close to lethal concentrations, while the second formulation showed little if any production. The lack of carbon monoxide production during pyrolyzation is the key difference in the survivability and toxicity of the two formulations.



# **Edemagenesis in F-344 Rats Exposed to SFE (Formulation A) Atmospheres**

**E C Kimmel<sup>1</sup>, E A Smith<sup>1</sup>, S Prues<sup>1</sup>, K Zepp<sup>1</sup>, MAJ J H English<sup>2</sup> and R L Carpenter<sup>3</sup>**

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Edemagenesis in F-344 rats exposed to atmospheres of pyrolyzed SFE (Formulation A) fire suppressant was evaluated by analyzing the enzyme activity in bronchoalveolar lavage (BAL) fluids and examining the respiratory tract tissues histopathologically. Twelve experimental groups of 12 animals each were exposed either to air or to 80 g/m<sup>3</sup> (aerosol phase) nominal concentrations of SFE for 1 hour. Exposures were conducted in a 700 L inhalation exposure chamber operated in a static mode. Exposure atmospheres were analyzed for actual aerosol mass concentration and size distribution, as well as the major gaseous components of SFE atmospheres, CO and CO<sub>2</sub>. Clinical observations were made at 15 minute intervals during the exposures. Time course of edemagenesis and resolution of the insult were determined by serial evaluation of animals at 1, 6, 12 and 24 hrs, as well as 2, 3, 4, 5, 6 and 7 days post exposure. Six animals from each group were used for analysis of BAL. These analyses included total protein, acid and alkaline phosphatase, lactate dehydrogenase, and  $\beta$ -glucuronidase enzyme determinations. BAL cellular component determination included cell number, viability and cell differential (polymorphonuclear leukocytes, pulmonary alveolar macrophages and lymphocytes). Histopathology performed on the remaining 6 animals per group included examination of nasal turbinates, pharyngeal, laryngeal, tracheobronchial and pulmonary (alveolar) tissues.

## **Evaluation of Blood Gas, pH, Hemoglobin, Bicarbonate and Glucose Levels after Exposure to the Pyrolyzed By-products of SFE Formulation A**

**E A Smith<sup>1</sup>, E C Kimmel<sup>1</sup>, S Prues<sup>1</sup>, J E Reboulet<sup>1</sup>,  
HMC S Bulger<sup>2</sup>, HM2 J Cassell<sup>2</sup> and R L Carpenter<sup>2</sup>**

### **1. GEO-CENTERS, INC.**

### **2. NAVAL MEDICAL RESEARCH INSTITUTE DETACHMENT (TOXICOLOGY)**

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Blood gases, pH, hemoglobin, bicarbonate ion and glucose were evaluated in F-344 rats during and after exposure to atmospheres of pyrolyzed SFE (Formulation A). Animals were randomized into 3 groups of 6 animals each, cannulated in the femoral artery and vein, and placed in nose-only holders located on the side of a 700 L whole-body inhalation chamber. Each group was exposed to either air (control), or a nominal concentration of 50 or 80 g/m<sup>3</sup> for 60 minutes under static conditions. Whole blood samples were collected at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 minutes. Blood samples were evaluated for pH, blood gases, bicarbonate ion, total hemoglobin, carboxyhemoglobin, methemoglobin, oxyhemoglobin, deoxyhemoglobin and glucose. Prior to the initiation of the main study two satellite studies were conducted: one examining the compatibility of blood types in 4 strains of rats, and the second examining the homeostasis of the animal during multiple blood collection/donation.

**PHYSICAL AND CHEMICAL CHARACTERISTICS OF  
SFE FIRE SUPPRESSANT ATMOSPHERES IN SMALL vs LARGE SCALE TESTS:  
Implications for Pulmonary Deposition and Toxicity.**

**E C Kimmel<sup>1</sup>, E A Smith<sup>1</sup>, J E Reboulet<sup>1</sup> and R L Carpenter<sup>2</sup>**

**1. Geo-Centers, Inc.**

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A comparison of the physical and chemical properties of SFE (a dry powder) fire extinguishant atmospheres was made between those generated to assess inhalation toxicity in a whole-body exposure system and those generated in facilities used to evaluate fire extinguishment efficacy. The purpose was to establish a basis for extrapolation of laboratory toxicity testing data to field conditions. Nominal concentrations of 50 and 80 g/m<sup>3</sup> in both systems were examined. Mass concentration and concentration decay of the aerosol component, aerosol size distribution and particle growth, and concentration profile of CO and CO<sub>2</sub>, the major gaseous components of SFE atmospheres, were determined. At comparable nominal concentrations, the initial mass concentration of SFE aerosols in the large system was 1.5 times greater than that in the exposure chamber. Aerosol concentration in both systems decayed exponentially. Hygroscopic growth of the particles was greater and more rapid in the large system. Gas concentrations in the larger test system also were greater by a factor of 1.5. However, in this system gas concentrations also underwent exponential decay whereas gas concentrations in the exposure chambers were steady state over the 1 hour test periods. Laboratory exposures at the higher nominal concentrations produced pulmonary edema and elevated COHb in rats. Theoretical, mathematical expressions (humans) characterizing CO<sub>2</sub> ventilatory effects on particle deposition and for COHb formation were derived as a basis for comparison of the potential toxicity of the atmospheres.

**Toxicological Evaluation of Exposure to Two Formulations of a  
Pyrotechnically-Generated Aerosol: Rangefinding and Multiple Dose**

**E A Smith<sup>1</sup>, E C Kimmel<sup>1</sup>, S Prues<sup>1</sup>, K Zepp<sup>1</sup>, J E Reboulet<sup>1</sup>,  
HMC S Bulger<sup>2</sup>, HM2 J Cassell<sup>2</sup> and R L Carpenter<sup>2</sup>**

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Fischer 344 rats (250-300 g) were exposed to the by-products of two formulations of SFE Formulation A, a pyrotechnically-generated aerosol fire extinguishant. Exposures levels ranged from 50 g/m<sup>3</sup> (nominal concentration) to 240 g/m<sup>3</sup>. The length of exposure was either 15 or 60 minutes under static conditions. A 700 L whole-body inhalation chamber was used for the exposures and consisted of a supply/exhaust system, aerosol generator and exhaust scrubber. The chamber atmosphere underwent aerosol [size (MMAD), distribution ( $\sigma_g$ ) and concentration] and gas [CO, CO<sub>2</sub> and O<sub>2</sub>] analysis. Clinical observations were taken throughout the exposure. Animals were euthanized 1-hr post-exposure and underwent histopathological examination and blood gas analysis. The difference in formulation caused dramatic results in survivability. The first formulation produced levels of carbon monoxide very close to lethal concentrations, while the second formulation showed little if any carbon monoxide. The lack of carbon monoxide production during pyrolyzation is the key difference in the survivability and toxicity of the two formulations.

**Determinates of SFE Toxicology:  
Interaction of Atmospheric Dynamic and Physiological Responses**

**R L Carpenter<sup>1</sup>, E C Kimmel<sup>2</sup> and E A Smith<sup>2</sup>**

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2. GEO-CENTERS, INC.**

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The physical and chemical properties of an aerosol have a direct influence upon fire suppression mechanisms and extinguishment efficacy. These same properties are also basic determinants of aerosol inhalation toxicity. To evaluate the toxicity of an aerosol, it must first be determined if the aerosol can be inspired. Particles with an median aerodynamic diameter of 1-2  $\mu\text{m}$  are capable of penetrating deep into the human tracheobronchial tree, reaching the alveolar acini. These acini are comprised of parenchymal tissues, which are highly susceptible to damage and least protected by clearance (defense) mechanisms. Both chemical and physical properties of inhaled particulate matter combine to control aerosol toxicity. The dose to regional lung tissue depends on particle size, particle specific surface area and chemical solubility. As is true of other routes of administration, tissue response to dissolved material in the lung is dependent on the chemical nature of the insult. Potential toxic effects include altered pulmonary function, irritation and altered gas exchange, leading to discomfort, altered oxygen uptake, incapacitation and death. In the case of pyrotechnically generated aerosols, carbon monoxide may also be present, resulting in increased carboxyhemoglobin levels and decreased oxygen uptake leading to disorientation and possible incapacitation. Aerosol toxic effects are not limited to the lung; dissolved material can be transported from the lung to other systems throughout the body. In addition to pulmonary and systemic effects, aerosolized material can cause irritation of the skin and/or nasal and ocular mucosa.

**EFFECTS OF TRIMETHYLOLPROPANE PHOSPHATE ON NEUROTRANSMITTER LEVELS IN THE RAT BRAIN. A. Jung\*, T.K. Narayanan\* and J. Rossi III. Naval Medical Research Institute Detachment-Toxicology and \*Geo-Centers Inc., Wright-Patterson Air Force Base, OH 45433-7903**  
**Sponsor: E. Smith**

Trimethylolpropane phosphate (TMPP), a pyrolytic product of synthetic lubricant MIL-L-23699, is a powerful convulsant causing generalized seizures in Fischer-344 rats at the dose level of 0.4 mg/kg. The molecular mechanism(s) underlying TMPP-induced seizures is uncertain. Competitive receptor binding studies have previously shown that TMPP has some affinity for the benzodiazepine receptor (Rossi et al., 1994). Using HPLC analysis, we measured neurotransmitter, metabolite, and excitatory and inhibitory amino acids levels in different brain regions of control and TMPP-treated rats. Statistically significant differences ( $p < 0.001$ ) in glutamic acid, glycine, and GABA levels in the brainstem ( $67.6 \pm 2.3 \mu\text{g}$ ;  $125.2 \pm 4.1 \mu\text{g}$ ;  $70.2 \pm 2.0 \mu\text{g}$ ), the caudal portion ( $198.6 \pm 5.3 \mu\text{g}$ ;  $31.7 \pm 1.0 \mu\text{g}$ ;  $93.0 \pm 4.3 \mu\text{g}$ ), and middle portion ( $205.5 \pm 4.8 \mu\text{g}$ ;  $28.9 \pm 0.4 \mu\text{g}$ ;  $110.0 \pm 2.1 \mu\text{g}$ ) of the brain were seen in the TMPP-treated rats. Minimal changes were found for catecholamines and their metabolites. The effects of TMPP were compared with those of the more well-studied convulsant, pentylenetetrazol (PTZ).

1. Sponsoring Agency.
2. IMMUNOLOGICAL ALTERATIONS BY ENDOCRINE DISRUPTORS
3. Human health effects.
4. Immunity, by definition, is a homeostatic condition in which the body maintains protection from infectious disease. It is a series of delicately balanced complex, multicellular, and physiological mechanisms that allow an individual to distinguish foreign material from "self" and to neutralize and/or eliminate the foreign matter. The complex nature of the immune system with its multiple humoral and cellular components makes it an easy target for many drugs and chemicals. Immunotoxicology is defined as an adverse response of the immune system to a chemical or drug which may result in a disease such as autoimmunity, immune suppression, allergy or other hypersensitive states. Occasionally, immune enhancement is the end result. The immunomodulating profiles of xenobiotics may be diverse, involving several components of the immune system or they may selectively compromise an individual compartment of the immune system. The following battery of tests will be carried out in assessing the immunotoxic potential of endocrine disruptors: (1) Using fluorescently labeled antibodies to cell surface markers in conjunction with flow cytometer, the effect of a toxicant on T cell population and its subset and on B cell population will be determined. (2) Phagocytic activity of the peritoneal macrophages from the exposed mice will be determined using 51-Cr labeled chicken red blood cells as phagocytic material. (3) Natural Killer (NK) cell activity will be evaluated by isolating NK cells from mouse spleen exposed to the toxicant with 51-Cr labeled YAC-1 tumor cell line as a target. (4) Acquired immunity (humoral) will be carried out by a plaque forming cell (PFC) assay, by harvesting the spleen cells from control and toxicant exposed mice after immunization with Sheep Red Blood Cell (SRBC) and incubating them with SRBC and complement. (5) Acquired immunity (cell mediated) will be measured using P815 mastocytoma as an allogenic target cell and immunizing the control and toxicant exposed mice. After sensitization, cytotoxic T lymphocytes from the spleen will be challenged with 51-Cr labeled P815 and the effector-to-target ratio will be compared with the control. The mouse has been the animal of choice because of the vast data base on its immune system and it is less expensive to maintain.
5. White KL. (1995) Scientifically based immunotoxicological testing strategies for chemicals (industrial and environmental) Hum. Exp. Toxicol. 14: ISS 1, 141-142.  
  
International workshop on environmental Immunotoxicology and human health. (1995)  
Hum. Exp. Toxicol. 14: ISS 1, 77-154.
6. Measurements
7. Hazard identification
8. ex vivo, mice
9. Immunological
- 10 PCBs
11. Naval Medical Research Institute Toxicology Detachment, 2612 Fifth street, Area-B, Bldg. 433, W.P.A.F.B., Ohio. 45433-7903.
12. Captain K.R. Still., K STILL @ NAVY.AL.WPAFB.AF.MIL.



1. Sponsoring agency
2. PEROXISOME PROLIFERATION BY ENDOCRINE DISRUPTORS
3. Human health effects
4. Diverse groups of chemicals which cause endocrine disruption such as herbicides, insecticides, industrial plasticisers, phthalate esters, and halogenated hydrocarbons are also peroxisome proliferators. Administration of these chemicals to rodents results in the dramatic proliferation of hepatic peroxisomes as well as liver hyperplasia. The process of xenobiotic-induced proliferation of peroxisomes in mammalian liver cells has received considerable attention because of the proposed relationship between induction of hydrogen peroxide producing peroxisomal enzymes and the development of liver cancer in mice and rats. It is currently believed that these chemicals are carcinogenic because of their ability to induce peroxisome proliferation. The carcinogenic potential of peroxisome proliferators occur mainly as the result of greater production of hydrogen peroxide due to increased  $\beta$ -oxidation of fatty acids leading to DNA damage or possibly tumor initiation. Peroxisome proliferators represent a good model system to study the mechanism(s) of non-genotoxic hepatocarcinogen. The induction of gene expression by peroxisome proliferators and by steroid hormones could share a common mechanism because both bind first to a binding protein and the resulting complex initiates transcription. Experimental evidence also shows that the peroxisome proliferator binding protein belongs to the steroid receptor superfamily. Peroxisome proliferation is a good indicator for screening chemicals in safety evaluation. The biochemical composition of hepatic peroxisomes includes the following enzymes: urate oxidase, D-amine oxidase, catalase, ATP dependent acyl-CoA synthetase. The bifunctional protein (~ Mr 80,000) characteristic of peroxisomes can be readily identified by SDS gel electrophoresis. These studies are designed to measure the enzyme activity and the bifunctional protein which are characteristic peroxisome markers in the rat liver after administration of the test compound.
5. Lock EA, Mitchell AM, and Elcombe CR. (1989) Biochemical Mechanisms Of Induction Of Hepatic Peroxisome Proliferation. *Annu. Rev. Pharmacol. Toxicol.* 29: 145-163  
  
Issemann I and Green S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. 347: 645-649
6. Method
7. Basic research
8. In vitro
9. Enzymology
10. Plasticisers
11. NMRI/TD, W.P.A.F.B., Ohio. 45433
12. Captain K. R. Still KStill@navy.al.wpafb.af.mil

1. Sponsoring Agency
2. MOLECULAR APPROACHES TO TOXICITY BY ENDOCRINE DISRUPTORS.
3. Exposure assessment
4. Molecular approaches have attained a central position in modern experimental and human toxicology by providing the means to study basic underlying mechanisms. Molecular methods can be used to identify casual associations that would otherwise be obscure and to make better quantitative estimates of these associations at relevant levels of exposure. The use of molecular approaches in toxicology should thus result in better estimates of risks to human health. Toxic effects of endocrine disruptors can be divided into two main classes on the basis of the mechanism of disease causation and reversibility of effects: traditional deterministic toxicity and stochastic toxicity. Deterministic toxicity overwhelms the body's compensatory processes, thereby exceeding the toxicity threshold and leading to pathological effects; the effects are however reversible, up to the late stages of the disease process. Toxic effects that arise from stochastic process have no clear threshold for toxicity, so that exposure to low doses of an agent over prolonged periods can give rise to serious effects that are not necessarily related to the level of exposure. Stochastic effects are irreversible or poorly reversible when low doses are given in the early stages of the disease. Most (if not all) molecular effects are stochastic; they occur as a result of a small number of irreversible changes in the information coded in DNA, resulting in mutagenesis, carcinogenesis and teratogenesis. Risk assessment is ultimately based on a quantitative estimate of the dose of the toxicologically relevant metabolite(s) that is delivered to target tissues. A new form of dosimetry is increasingly being attempted that is based on the analysis of the products of chemicals with macromolecules in vivo. Our studies include the measurement of DNA adducts from the target tissues by (1) fluorescence spectroscopy, (2) the use of monoclonal and polyclonal antibodies, (3) 32P- labeling followed by HPLC separation. Hemoglobin adducts in the exposed animals will also be carried out, since there is increasing acceptance that hemoglobin adducts are a relevant dosimeter of the exposed population.
5. ECETOC. ( 1989) DNA and protein adducts: evaluation of their use in exposure monitoring and risk assessment. Monograph no 13. Brussels; European Chemical Industry Toxicology Center. 1-89  
  
Shuker DEG. ( 1989) Nucleic acid-carcinogen adducts in human dosimetry. *Arch. Toxicol.* 13: 55-65
6. Measurements
7. Exposure and risk model
8. In vitro
9. Carcinogenesis
- 10 Alkyl phenols
- 11 Naval Medical Research Institute/ Toxicology Detachment, 2612 Fifth street, Area- B, Bldg. 433  
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1. SPONSORING ORGANIZATION: Naval Medical Research Institute Detachment (Toxicology)
2. TITLE: Cytochrome P450 Technology For Assessing The Safety And Efficacy Of DBNP.
3. PROJECT FOCUS: Human Health Effects

4. BRIEF DESCRIPTION: Studies of xenobiotic biotransformation by *in vitro* techniques are destined to play an increasingly important role in assessing the safety and efficacy of drugs and other chemical entities. Different chemical classes of compounds cause endocrine disturbance either through the parent chemical or the reactive metabolite(s) or both. The liver plays the central role in metabolizing xenobiotics with its numerous cytochrome P450 enzymes. The liver microsomal P450 enzymes involved in xenobiotic biotransformation belong to 4 main gene families, namely, SPONSORING CYP1, CYP2, CYP3, and CYP4. Without exception, the levels and activity of each P450 enzyme have been shown to vary from one individual to the next due to environmental and/or genetic factors. Exposure to environmental factors or xenobiotics can induce or block the P450 enzyme synthesis or can activate or inactivate the pre-existing enzyme. This can lead to altered biotransformation of the parent compound as well as chemicals of different classes because of broad and often overlapping substrate specificity of liver microsomal P450 enzymes resulting in exaggerated pharmacological or toxicological responses. Evaluating the effect of endocrine disrupters on the P450 enzyme profile, one can predict pharmacokinetic tolerance, drug interaction, carcinogenic potential and toxicity. Our studies are designed to measure the following characteristic enzyme reactions of each gene family: (1) CYP1- Benzo[a]pyrene-3-hydroxylation; (2) CYP2- testosterone 16 $\beta$ -hydroxylation; (3) CYP3 - erythromycin N-demethylation; and (4) CYP4- lauric acid 12-hydroxylation in **a.** by an *ex vivo* procedure whereby the xenobiotic is administered to rats or mice *in vivo*, followed by measurement of P450 enzymes in liver microsomal preparation *in vitro*, **b.** in precision-cut rat liver slices, and **c.** in a rat liver cell line.

5. SELECTED REFERENCES:

Parkinson A. ( 1996) An overview of current cytochrome P450 technology for assessing the safety and efficacy of new materials. *Toxicologic Pathology*. 24 : 45-57

6. KEYWORD FOR INVENTORY CATEGORY FROM NEEDS DOCUMENT:

## FY98 NMRI/TD PREPROPOSAL

1. **Date Prepared:** November 25, 1996
2. **Title of the Project:** Further Development of the NTAS: Cytokines as a biomarker for the processes underlying non-insult related performance decrement.
3. **Principal Investigator:** (1) John Rossi III  
(1) CDR(S.), Ph.D.
4. **Position Title:** Principal Investigator

***Laboratory:*** NMRI/TD, WPAFB, OH  
***Department:*** Neurobehavioral Toxicology  
***Phone Number (Comm):*** (937) 255-6058  
***(DSN):*** 986-7094  
***FAX Number (Comm):*** (937) 656-7094  
***(DSN):*** 986-7094  
***E-mail Address:*** (1) jrossi@navy.al.wpafb.af.mil

5. **Dates of En. Work Period:** 01 Oct. '97 - 30 Sept. '00

6. **Resources:**

- a. ***Funding Category:*** (6.2)

- b. ***Funding Required for each Fiscal Year and Total Funds:***

Y1:	\$70K
Y2:	\$80K
Y3:	\$80K
<b>Total:</b>	<b>\$230K</b>

- c. ***Personnel Required:***

Professional (contract):	1 man-year/yr
Professional (contract):	1 man-year/yr

- d. ***Equipment Required:*** None

7. **Navy Needs:**

(a) Performance decrement as a result of exposure to toxicants is of great concern to the operational Navy. NMRI/TD has successfully developed (1991-1995) the Navy Neurobehavioral Toxicity Assessment Battery (the NTAB) to provide a comprehensive tool for identifying subtle performance deficits arising from acute or long-term exposure to low levels of toxicants. Concurrently, NMRI/TD has been involved in the development, testing and validation of the Navy Neuro-Molecular

Toxicity Assessment System (the NTAS). The NTAS includes a battery of cellular-level tests to identify toxicant-induced insults to the CNS that may correlate with, and ultimately predict, gross performance deficits. Some deficits, however, do not appear to involve histopathological insults that can be identified with existing technology. An example of "non-insult" related neurotoxicity is the long-term CNS sensitization effects shown at NMRI/TD to follow a single low dose administration of trimethylolpropane phosphate (TMPP). A single dose of TMPP as low as 0.0125 mg/kg has been shown to increase audiogenic seizure susceptibility and alter social interaction in rats as long as 90 days post-administration. Extensive metabolic and histopathology studies have failed to recognize any TMPP or related metabolites in body tissues (beyond 96 hr post-administration), or identify any cellular-level tissue changes in rats "sensitized" by the TMPP exposure.

There is mounting evidence that some performance deficits may result from toxicant-induced changes in the level of certain endogenous compounds involved in body regulation, without induction of detectable histopathology. Cytokine expression in the CNS (IL-1, IL-2, IL-4, *etc.*), in response to toxicant or stress exposure, may be both an adequate predictive biomarker of neurotoxicity, as well as a possible causative factor in non-insult neurotoxicity.

(b) This research will determine if cytokine presence in the CNS is a suitable biochemical marker for the processes underlying subtle performance decrements. Once established, the system will be included in the NTAS, providing valuable information concerning a possible relationship between cytokine production and neurobehavioral performance decrement.

(c) Official Requirements:

OPNAVINST 4110.2	Hazardous Materials Control & Management
OPNAVINST 5100.23B	Occupational Safety and Health
OPNAVINST 5090.1	Environmental Protection
Science and Technology Roundtable Guidance	

## 8. Specific Aims:

Predicting the performance decrement caused by a toxicant and understanding the neuro-molecular mechanism(s) of degradation as a function of changes in neuroanatomy, neurochemistry or neurophysiology is important to understanding its neurotoxicity. If one can identify mechanisms by which toxic insult occurs, then treatment strategies may be developed to counteract the toxin. The rationale behind selecting cytokines as a suitable biomarker for the prediction of performance decrement is as follows:

- (1) There is interaction between the nervous, endocrine, and immune systems in the body
- (2) Cytokines have been postulated to effect all three systems
- (3) Nervous, endocrine, and immune systems have receptors for cytokines

The specific aim of the proposed research program is to identify specific cytokines as predictors of non-insult related performance decrement caused by toxicants. Cytokines to be investigated include:

Interlukin 1, 2, 4, and 6 (IL-1, IL-2, IL-4 and IL-6)  
Tumor Necrosis Factor (TNF)  
 $\alpha$ -Interferon ( $\alpha$ -IFN)

The cytokines were selected for study due to their known effect on the hypothalamic-pituitary-adrenal axis and role in neurobehavior (Ref. 1-6).

## 9. Experimental Design and Methods:

(a) The Navy Neurobehavioral Toxicity Assessment Battery (NTAB), developed at NMRI/TD, is being evaluated in its application to predict human behavioral toxicity. The NTAB was developed to allow assessment of partial or complete incapacitation in eight elemental neuroaxis subsystems of behavior. Animals in this proposal will be exposed to well-understood neurotoxicants (*e.g.* TMPP) and analyzed for performance decrements with selected tests from the NTAB. Toxicant-induced cytokine upregulation will then be correlated with performance decrement.

(b) Cytokines will be quantitated using commercially available standard assay kits and the results will be subjected to statistical analysis.

### (c) *Associate Investigators:*

- |   |   |
|---|---|
| (1) Tanjore K. Narayanan (Geo-Centers)  | Biochemical Analysis  |
| (2) Glenn Ritchie, Ph.D. (Geo-Centers): | Application of data and behavioral analysis                       |
| (3) Anne Jung, MS. (Geo-Centers):       | Neurochemistry, pharmacokinetic analysis, ligand binding analysis |
| (4) Alan Nordholm, Ph.D. (USNR)         | Behavioral pharmacology   |

## 10. Assessment of Risk:

The perceived level of risk of the research is low. Equipment and personnel necessary to carry out this project are in place at NMRI/TD.

## 11. Related activities:

(a) Cytokine research is conducted at numerous other laboratories. There is, however, no current research correlating toxicant-induced cytokine expression with subtle changes in behavioral performance.

(b) DTIC, Medline and Toxline database searches have all been conducted. No similar literature was identified to the research proposed in this document.

(c) Our laboratory routinely interacts with other government laboratories and educational research institutions. For example, work has been contracted at both Wright State University, Dayton, OH and Bowling Green State University, Bowling Green, OH.

#### **12. Transition Approach:**

The interface between the research program and the fleet would occur through the Naval Environmental Health Center (NHEC), the agency responsible for coordinating all fleet toxicity issues. It is anticipated that through this research and research from follow-on proposals, the Navy will obtain a unique set of biomarkers for determining the potential for subtle toxicity consequences from exposure to Navy relevant compounds.

#### **13. Literature Cited (6):**

- (1) Hermus, A.R. & Sweep, C.G. (1990). Cytokines and the hypothalamic-pituitary-adrenal axis. *Journal of Steroid Chemistry and Molecular Biology*, 37, 867-871.
- (2) Besedovsky, H., del Rey, A., Sorkin, E., Da Parda, M., Burri, R., & Honegger, C. (1983). The immune response evoke changes in brain noradrenergic neurons. *Science*, 221, 564-566.
- (3) Naitoh, Y., Fukata, J., Tominaga, T., *et al.* (1988). Interlukin-6 stimulates the secretion of adrenocorticotrophic hormone in conscious, freely moving rats. *Biochem. Biophys. Res. Commun.* 155, 1459-1463.
- (4) Birmanns, B., Saphier, D., & Abramsky, O. (1990).  $\alpha$ - Interferon modifies cortical EEG activity: Dose dependency and antagonism by naloxone. *Journal of the Neurological Sciences*, 100, 22-26.
- (5) Saphier, D. (1992). Electrophysiological effects of cytokines in relation to arousal and adrenocortical secretion. In N.J. Rothwell & R. Dantzer (Eds), *Interlukin-1 in the Brain*, Oxford, UK: Pergamon Press, pp. 51-73.
- (6) Saphier, D. Ovadia, H., & Abramsky, O. (1990). Neural responses to antigenic challenges and immunomodulatory factors. *Yale Journal of Biology and Medicine*, 63, 109-119.

#### **14. Suggested Reviewers (2):**

- (1) Jaak Panksepp, Ph.D., Dept. of Psychology, Bowling Green State University, Bowling Green, OH 43403, (419) 372-2819
- (2) Michael J. Forster, Ph.D., Dept. Pharmacol., Texas College of Osteopathic Medicine, 3500 Camp Bowie Blvd., Fortworth, TX 76107, (817) 735-2092



## FY98 NMRI/TD PREPROPOSAL

1. **Date Prepared:** November 25, 1996
2. **Title of Project:** Further Development of the NTAS: Steroids and oxidative stress
3. **Principal Investigators:**  
(1) Alan F. Nordholm  
(1) LT, Ph.D.
4. **Position Title:** (1) Principal Investigator  
  
*Laboratory:* NMRI/TD, WPAFB, OH  
*Department:* (1) Neurobehavioral Toxicology  
*Phone Number (Comm):* (937) 255-6058  
*(DSN):* 986-7094  
*FAX Number (Comm):* (937) 656-7094  
*(DSN):* 986-7094  
*E-mail Address:* (1) anordholm@navy.al.wpafb.af.mil
5. **Dates of En. Work Period:** 01 Oct. '97 - 30 Sept. '00

### 6. **Resources:**

- a. ***Funding Category:*** (6.2)
- b. ***Funds Required for each Fiscal Year and Total Funds:***  
Y1: \$60K  
Y2: \$70K  
Y3: \$70K  
Total: \$200K
- c. ***Personnel Required:***  
Professional (contract): 1 man-year/yr  
Professional (contract): 1 man-year/yr
- d. ***Equipment Required:*** None

### 7. **Navy Need:**

(a) Navy personnel, whether in combat or daily operational roles, are subjected to various degrees of environmental, chemical or psychological stress. It is well known that stress can induce changes in the capacities of both animals and humans, resulting in possible performance decrement. Stress-induced changes in the performance capacity of military personnel can result in a reduction in manpower efficiency, mission failure and loss of life. The neuro-molecular mechanisms underlying stress-induced changes in behavioral performance are, however, poorly understood. Likewise, it is unknown whether the reliable biomarkers of stress (*i.e.*, corticosterone levels, corticotropin releasing

factor, forebrain c-Fos, heat shock protein, *etc.*) are actually involved in the physiological processes underlying acute and long-term stress responses.

(b) NMRI/TD has successfully developed (1991-1995) the Navy Neurobehavioral Toxicity Assessment Battery (the NTAB) to provide a comprehensive tool for identifying subtle performance deficits resulting from environmental toxicants and stressors. Concurrently, NRMI/TD has been involved in the development, testing and validation of the Navy Neuro-Molecular Toxicity Assessment System (the NTAS). The NTAS includes a battery of cellular-level tests to identify toxicant- and stress-induced insults to the CNS that may correlate with, and ultimately predict, gross performance deficits. Presently, the NTAS does not include test methodology to: (a) identify known biomarkers associated with stress and (b) investigate stress-induced neuro-molecular changes that may result in permanent changes in histopathology.

(c) Several lines of evidence have linked reactive oxygen species (ROS) with a wide variety of pathological events, such as atherosclerosis (1), cancer (2), aging (3), Parkinson's disease (4), diabetic complications (5), and central nervous system injury (6). There is also a wide literature on the effects of stress and serum cortisol levels. This proposal seeks to link the increases in cortisol levels with ROS and performance decrement. It is anticipated, that using the techniques described in this proposal, the Navy will gain insights performance decrements associated with oxidative stress.

(d) Official Requirements:

OPNAVINST 4110.2	Hazardous Materials Control & Management
OPNAVINST 5100.23B	Occupational Safety and Health
OPNAVINST 5090.1	Environmental Protection
Science and Technology Roundtable Guidance	

## 8. Specific Aims:

(a) Reactive oxygen species (ROS) are generated as a byproduct of the redox reaction taking place in the cell during respiration, normal metabolism, metabolism of xenobiotics, or stress. Under physiological conditions, the ROS are scavenged by antioxidants. Oxidative stress denotes a shift in the prooxidant/antioxidant balance in favor of the prooxidants, with possible resultant cell damage. The hypothesis of this proposal is that subtle performance decrements seen in individuals under stress may result from histopathological changes induced by oxidative stress.

(b) The end result of this research will be development of test methodology to predict behavioral performance decrement as a result of oxidative stress. This will be done by measuring cortisol levels and free-radical scavengers. This methodology will be incorporated in the NTAS.

## 9. Experimental Design and Methods:

(a) Experiments will be carried out in both young and older rats (of both sexes), as well as in adrenalectomized rats. Adrenalectomized rats are required to control for the effects of endogenous corticosteroids. Animals will be exposed to one of several stressors (*e.g.*, mechanical, heat, physical, chemical). Rats will then be evaluated on tests selected from the Navy Neurobehavioral Toxicity

Assessment Battery to determine any performance deficits induced by the aforementioned stressors. This will be followed by a battery of biochemical tests to include detection for :

- |   |                         |
|---|-------------------------|
| (1) Reactive oxygen species, using chemical trap. | (5) Antioxidant enzymes |
| (2) Level of corticosteroid in the blood          | (6) Antioxidants        |
| (3) Lipid peroxidation                            | (7) DNA adducts         |
| (4) Carbonyl proteins                             |                         |

(b) It is ethically and procedurally impossible to perform the experiments contained in this proposal on humans. The only viable approach is study the effects of these stress in animals and develop a model which will extrapolate to humans.

(c) *Associate investigators:*

- |  |   |
|--|---|
| (1) Tanjore K. Narayanan (Geo-Centers) | Biochemical analysis  |
| (2) Anne Jung MS (Geo-Centers)         | Enzyme assays, HPLC analysis,<br>gel-electrophoresis<br>Tissue culture. |

**10. Assessment of Risk:**

(a) The perceived level of risk of the research is low. The equipment and personnel necessary to carry out the experiments contained in this preproposal are in place at WPAFB.

**11. Related Activities:**

(a) No other government groups are actively engaged in neurobehavioral toxicology research at the same level as is NMRI/TD.

(b) DTIC, Medline and Toxline database searches have all been conducted. No similar literature was identified to the research proposed in this document.

(c) Our laboratory routinely interacts with other government laboratories and educational research institutions. For example, work has been contracted at both Wright State University, Dayton, OH and Bowling Green State University, Bowling Green, OH.

**12. Transition Approach:**

The interface between the research program and the fleet would be through the Naval Environmental Health Center (NHEC), the agency responsible for coordinating all fleet toxicity issues.

It is anticipated that through this research and follow-on proposals the Navy will obtain a unique set of biomarkers for determining performance decrements as they relate to oxidative stress.

**13. Literature Cited (6):**

- (1) Carew, T.E., Schwenke, D.C., & Steinberg, D. (1987).** Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA*, **84**, 7725-7729
- (2) Weisburger, J.H. (1991).** Nutritional approach to cancer prevention with emphasis on vitamins, antioxidants and carotenoids. *Am. J. Clin. Nutr.*, **53**, 226S-237S.
- (3) Harman, D. (1982).** The free radical theory of aging. In Pryor, W.A., ed. *Free radicals in biology* (Vol. 5). New-York., Academic Press., 255-275.
- (4) Gerlach, M., Ben-Shachar, D., Riederer, P., & Youdim, M.B.H. (1994).** Altered brain metabolism of iron as a cause of neurodegenerative disease? *J. Neurochem.* **63** (No. 3), 793-807.
- (5) Parthiban, A., Vijayalingam, S., Shanmugasundaram, K.R., & Mohan, R. (1995).** Oxidative stress and the development of diabetic complications- Antioxidants and lipid peroxidation in erythrocytes and cell membrane. *Cell. Biol. Intern.*, **19** (No. 12), 987-993.
- (6) Carney, J.M., & Carney, A.M. (1994).** Role of protein oxidation in aging and in age-associated neurodegenerative diseases. *Life. Sci.* **55** (No. 25/26), 2097-2103.

**14. Suggested Reviewers (2):**

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# THE EFFECT OF A TOXIN ON CELL CULTURES

by

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In this report we describe results of the analysis of data obtained from experiments which subject live cell cultures to differing concentrations of acetoaminophenol. We also present a mathematical model which exhibits behavior similar to that of the experiments.

## 1. Experiments 4 and 6

Cells are grown in a culture medium on a plate until they are confluent. The cells are exposed to acetoaminophenol at different concentrations (mg/ml of culture medium). In experiment 6, the ABS is measured after 3 hours. In experiment 4 the ABS is measured after 24 hours. The data are displayed in Table 1. There are three replications for each treatment group.

The ABS measure is converted to number of viable cells by the formula

$$\# \text{ cells} = (1.19 \times 10^5) + (4.59 \times 10^6 \times \text{ABS}) + (6.18 \times 10^5 \times \text{ABS}^2). \quad (1.1)$$

**Table 1**  
Data From Experiments 4 and 6  
Cell exposure to acetoaminophenol on confluent plates

Dose mg/ml of culture medium	Exposure Time (hrs)	ABS for each replicate		
		1	2	3
0	24	0.763	0.737	0.733
1	24	0.693	0.606	0.702
2	24	0.732	0.719	0.737
4	24	0.418	0.586	0.490
6	24	0.055	0.067	0.031
8	24	0.009	0.010	0.009
0	3	0.7625	0.8019	0.7697
1	3	0.7458	0.79602	0.73566
2	3	0.7364	0.7421	0.7504
4	3	0.1684	0.8162	0.7573
8	3	0.4755	0.4006	0.7330
16	3	0.0039	0.0066	0.0035
# of cells = $(1.19 \times 10^5) + (4.59 \times 10^6 \times \text{ABS}) + (6.18 \times 10^5 \times \text{ABS}^2)$				

Figures 1 – 3 display the data and the results of fitting a logistic regression with number of trials  $4.3 \times 10^6$  and covariates a constant and dose. The parameter estimates appear in Tables 2 – 3.

**Table 2**  
Experiment 6: Exposure Time = 3 hours

$$\text{Model: } E[\# \text{ viable cells}] = \frac{4.3 \times 10^6 \exp\{\beta_0 + (\beta_1 \times \text{dose})\}}{1 + \exp\{\beta_0 + (\beta_1 \times \text{dose})\}}$$

Data	Parameter Estimate (Standard Error)	
	$\beta_0$	$\beta_1$
All	3.32 (0.0007)	-0.37 (0.00008)
without repl 1 for dose 4	2.77 (0.0005)	-0.33 (0.00008)

## 2. A Mathematical Model

In this section we present a system of differential equations whose solution exhibits a similar shape to that of the data of experiments 4 and 6. We adapt an account by Ballantyne *et al.* (1993) of the action of paracetamol=acetoaminophenol to cause hepatic cell necrosis in a dose-responsive way.

There are three pathways of metabolism for acetoaminophenol. One of the pathways involves an initial reaction with an enzyme P-450 to form a toxic metabolite. This toxic metabolite can be metabolized by glutathione or by the cells. The metabolite is toxic to the cells.

Let  $E(t)$  be the amount of enzyme present at time  $t$ . Let  $B(t)$  be the amount of acetoaminophenol present outside the cells at time  $t$ . Let  $A(t)$  be the amount of acetoaminophenol present in the cells at time  $t$ . Let  $G(t)$  be the amount of glutathione present at time  $t$ . Let  $C(t)$  be the number of viable cells at time  $t$ . Our model is as follows.

$$E(t) = k_{CE}C(t); \quad (2.1a)$$

the enzyme present is proportional to live cells;

$$G(0) = k_{GMC}C(0); \quad (2.1b)$$

the amount of glutathione present at time 0 is proportional to the number of live cells at time 0;

$$\frac{dM(t)}{dt} = k_{EA}K_{EA}^p \frac{E(t)(A(t)/K_{EA})^p}{1 + (A(t)/K_{EA})^p} - k_{GM}G(t)M(t); \quad (2.1c)$$

the rate of change in metabolite decreases in proportion to the product of the amount of glutathione and the amount of metabolite; it increases in proportion to



the interaction between enzyme and acetoaminophenol, saturating at a value proportional to  $E(t)$  for large values of  $A(t)$ ;

$$\frac{dA(t)}{dt} = -k_{OA} \frac{A(t)}{1 + A(t)/K_{OA}} - k_{EA} K_{EA}^p \frac{E(t)(A(t)/K_{EA})^p}{1 + (A(t)/K_{EA})^p}; \quad (2.1d)$$

$$+ k_{BA} C(t) B(t) + k_{GMA} k_{GM} G(t) M(t)$$

the rate of change in the amount of acetoaminophenol inside the cell decreases due to other metabolic pathways and saturates for large values of  $A(t)$ ; it also decreases in proportion to the product of the amount of enzyme present and the amount of acetoaminophenol inside the cells saturating at a value proportional to  $E(t)$  for large  $A(t)$ ; it increases at a rate proportional to the product of the cells present and the amount of acetoaminophenol outside the cells; it also increases in proportion to the product of the amount of glutathione and amount of the metabolite; this latter increase occurs when the metabolite oxidizes glutathione which results in the metabolite being itself reduced back to acetoaminophenol;

*assuming steady state  
x port 9 es  
tion initial  
cell. Cool  
write sol  
driven me  
x port 9.*

$$\frac{dC(t)}{dt} = -k_{MC} M(t) C(t) + k_N C(t) \max \left[ 0, 1 - \frac{C(t)}{C_0} \right]; \quad (2.1e)$$

*True far away from threshold.*

the cell kill rate is proportional to the product of cells present and the current metabolite concentration level; the cell growth rate is logistic where  $C_0$  is a constant.

*Vary the fa  
of 2.1e to  
growth  
constant  
"Drive  
NEXT-  
Signaling  
Mode.*

$$\frac{dG(t)}{dt} = -k_{GM} G(t) M(t) - (k_{MC} k_{GMC} M(t) C(t)) I\{G(t) > 0\}; \quad (2.1f)$$

the amount of glutathione present decreases in proportion to the value of the metabolite and amount of glutathione; it also decreases in proportion to the rate at which cells are killed;  $I\{G(t) > 0\} = 1$  if  $G(t) > 0$  and 0 otherwise;

$$\frac{dB(t)}{dt} = -k_{BA} C(t) B(t);$$

*1/2 cell death  
deaths  
glutathione  
(2.1g)*

*True during exposure.*

the amount of acetoaminophenol present outside the cells decreases in proportion to the product of the amount outside the cells and the number of cells present.

Equations (2.1a)-(2.1g) were solved numerically using the 4th/5th order Runge-Kutta-Fehlberg method implemented in MATLAB. Figure 4 presents a graph of the fraction of viable cells at time  $t = 3$  versus dose. Figure 5 displays the fraction of cells that are viable versus dose for a model with the same parameters as that for Figure 4 but using  $p = 2$  (rather than  $p = 1$  of Figure 4). Figure 6 displays the fraction of viable cells for the model with the same parameters except  $p = 3$ . Note that increasing  $p$  results in the curve for the number of viable cells becoming more S-shaped.

In the equations (2.1), the toxic metabolite can kill cells as expressed in equation (2.1e) but the metabolite is unaffected by the cell deaths. By modifying equation (2.1c) to

$$\frac{dM(t)}{dt} = k_{EA}K_{EA}^p \frac{E(t)(A(t)/K_{EA})^p}{1 + (A(t)/K_{EA})^p} - k_{GM}G(t)M(t) - k_{MC}M(t)C(t) \quad (2.2c)$$

we are assuming that cells metabolize the metabolite but are killed in the process. Figure 7 displays a graph of the fraction of viable cells for  $p = 1$  obtained by solving the system of equations (2.1a) – (2.1f) with (2.2c) replacing (2.1c), and the other parameters the same as the other graphs. The shape of the curve is about the same. However, there is an increase in the fraction of viable cells. Figure 8 displays a graph of the fraction of viable cells for  $p = 2$ , with the other parameters the same.

*High dose survivability =*

### 3. Experiment 7

In experiment 7, confluent plates are dosed for 3 hours with acetoaminophenol and then the acetoaminophenol is removed. The ABS is measured at times 0, 24, 48, and 72 hours after removal. Table 4 displays the data. Figures 9 – 11 display the data by dosage and time. In this experiment the number of cells is obtained from the ABS using the following formula

$$\# \text{ of cells} = (1.21 \times 10^5) + (1.75 \times 10^6 \times \text{ABS}) - (1.32 \times 10^5 \times \text{ABS}^2).$$

An ANOVA for the control data indicates that the mean number of cells at different times for the control is significantly different ( $F = 4.87$ ,  $df_B = 3$ ,  $df_W = 16$ ,

**Table 4**  
Data From Experiment 7  
Confluent plates were dosed for 3 hours and then  
the acetoaminophenol was removed.

Dose mg/ml of culture medium	Time (hrs) after acetoami- nophenol was removed	ABS for each replicate						Median
		1	2	3	4	5	6	
0	0	0.695	0.715	0.720	0.730	0.713	0.728	0.717
0	24	0.792	0.773	0.788	0.810	–	–	0.790
0	48	0.799	0.823	0.803	0.781	–	–	0.801
0	72	0.745	0.696	0.884	0.840	0.775	0.756	0.766
6	0	0.617	0.610	0.642	0.652	0.647	0.653	0.645
6	24	0.672	0.720	0.715	0.677	–	–	0.696
6	48	0.770	0.759	0.828	0.783	–	–	0.776
6	72	0.828	0.733	0.811	0.762	0.804	0.769	0.786
8	0	0.389	0.379	0.496	0.478	0.423	0.411	0.417
8	24	0.387	0.369	0.378	0.398	–	–	0.382
8	48	0.449	0.440	0.418	0.428	–	–	0.434
8	72	0.440	0.414	0.550	0.524	0.704	0.654	0.537

The ABS measure is converted to number of viable cells by the formula

$$\# \text{ of cells} = (1.21 \times 10^5) + (1.75 \times 10^6 \times \text{ABS}) - (1.32 \times 10^5 \times \text{ABS}^2)$$

Table 5 displays data from the experiment. The number of viable cells is obtained from the ABS measure using (1.1).

In experiment 5, confluent plates are dosed with acetoaminophenol at a concentration of 8mg/ml of culture medium and exposed for differing times. Table 6 displays data from the experiment. The number of viable cells is obtained from the ABS measure using (1.1).

**Table 5**  
Data From Experiment 3  
Cells confluent before being dosed  
Acetoaminophenol at a concentration of 6mg/ml culture medium

Exposure Time (hrs)	ABS for each replicate			
	1	2	3	4
8	0.918	1.027	0.865	0.949
12	0.494	—	0.601	0.576
16	0.531	0.474	0.616	0.313
25	0.012	0.014	0.004	0.017

The ABS measure is converted to number of viable cells by the formula  

$$\# \text{ of cells} = (1.19 \times 10^5) + (4.59 \times 10^6 \times \text{ABS}) + (6.18 \times 10^5 \times \text{ABS}^2)$$

**Table 6**  
Data From Experiment 5  
Cells confluent before being dosed  
Acetoaminophenol at a concentration of 8mg/ml culture medium

Exposure Time (hrs)	ABS for each replicate		
	1	2	3
2	0.773	0.817	0.817
4	0.091	0.090	0.321
6	0.047	0.040	0.145
8	0.045	0.010	0.028
24	0.006	0.005	0.008
Control			
24	0.743	0.78	0.774

The ABS measure is converted to number of viable cells by the formula  

$$\# \text{ of cells} = (1.19 \times 10^5) + (4.59 \times 10^6 \times \text{ABS}) + (6.18 \times 10^5 \times \text{ABS}^2)$$

Figure 12 displays data from experiment 3; it also displays the results of fitting a logistic regression model to the data. The logistic regression model and parameter estimates are displayed in Table 7.

Figure 13 displays data from experiment 5; it also displays the results of fitting a logistic regression model to the data. The logistic regression model and parameter estimates are displayed in Table 8.

**Table 7**  
Experiment 3  
Confluent cells are exposed to acetoaminophenol  
at concentration 6mg/ml culture medium

$$\text{Model: } E[\# \text{ viable cells}] = (5.5 \times 10^6) \frac{\exp\{\beta_0 + (\beta_1 \times \text{Time})\}}{1 + \exp\{\beta_0 + (\beta_1 \times \text{Time})\}}$$

Parameter Estimates (Standard Error)	
$\beta_0$	$\beta_1$
4.22	-0.29
(0.0009)	(0.00007)

**Table 8**  
Experiment 5  
Confluent cells are exposed to acetoaminophenol  
at concentration 8mg/ml culture medium (without control)

$$\text{Model: } E[\# \text{ viable cells}] = (4.3 \times 10^6) \frac{\exp\{\beta_0 + (\beta_1 \times \text{Time})\}}{1 + \exp\{\beta_0 + (\beta_1 \times \text{Time})\}}$$

Parameter Estimates (Standard Error)	
$\beta_0$	$\beta_1$
2.15	-0.606
(0.0008)	(0.0002)

The logistic regression models, displayed in Tables 7 and 8 can be used to estimate the time until 50% of the cells are killed.

To find an estimate of  $T_{50}$ , the time until 50% of the cells are killed, set

$$\frac{\exp\{\hat{\beta}_0 + \hat{\beta}_1 t\}}{1 + \exp\{\hat{\beta}_0 + \hat{\beta}_1 t\}} = 0.5$$

and solve for  $t$

$$\hat{t}_{50} = -\frac{\hat{\beta}_0}{\hat{\beta}_1}.$$

An asymptotic variance for  $\hat{t}_{50}$  is

$$\begin{aligned} \text{Var}(\hat{t}_{50}) = & \left(\frac{1}{\hat{\beta}_1}\right)^2 \text{Var}[\hat{\beta}_0] + \left(\frac{\hat{\beta}_0}{\hat{\beta}_1^2}\right)^2 \text{Var}[\hat{\beta}_1] \\ & - 2\left(\frac{1}{\hat{\beta}_1}\right)\left(\frac{\hat{\beta}_0}{\hat{\beta}_1^2}\right) \text{Cov}[\hat{\beta}_0, \hat{\beta}_1] \end{aligned}$$

where  $\text{Var}[\hat{\beta}_0]$ ,  $\text{Var}[\hat{\beta}_1]$  and  $\text{Cov}[\hat{\beta}_0, \hat{\beta}_1]$  are obtained from the estimated variance-covariance matrix (cf. Morgan [1992], p. 61).

For the data from experiment 3 (dose 6mg/ml),  $\hat{t}_{50} = 14.34$  with standard error of 0.001. For the data from experiment 5 (dose 8mg/ml),  $\hat{t}_{50} = 3.55$  with standard error 0.0006.

Figure 14 displays data from experiments 3 and 5 and the results of fitting one logistic regression model to both sets of data without the control data from experiment 5. The regression model appears in Table 9.

**Table 9**  
Experiments 3 and 5  
Confluent cells exposed to acetoaminophenol (without control)

$$\text{Model: } E[\# \text{ viable cells}] = \frac{(5.5 \times 10^6) \exp\{\beta_0 + (\beta_1 \times \text{Time}) + (\beta_2 \times \text{Dose}) + (\beta_3 \times \text{Dose} \times \text{Time})\}}{1 + \exp\{\beta_0 + (\beta_1 \times \text{Time}) + (\beta_2 \times \text{Dose}) + (\beta_3 \times \text{Dose} \times \text{Time})\}}$$

Parameter Estimates (Standard Errors)			
$\beta_0$	$\beta_1$	$\beta_2$	$\beta_3$
12.88	0.39	-1.44	-0.11
(0.004)	(0.0006)	(0.0006)	(0.00009)

Table 10 displays parameter estimates for the logistic regression whose parameters are estimated using data for positive dosages from experiments 3 and 5 along with the control data from experiment 5.

**Table 10**  
Experiments 3 and 5  
Confluent cells exposed to acetoaminophenol  
(with control from experiment 5)

$$\text{Model: } E[\# \text{ viable cells}] = \frac{(5.5 \times 10^6) \exp\{\chi\beta\}}{1 + \exp\{\chi\beta\}} \text{ where}$$

$$\chi\beta = \beta_0 + (\beta_1 \times \text{Time}) + (\beta_2 \times \text{Dose}) + (\beta_3 \times \text{Dose} \times \text{Time})$$

Parameter Estimates (Standard Errors)			
$\beta_0$	$\beta_1$	$\beta_2$	$\beta_3$
20.17	-0.80	-2.51	0.072
(0.004)	(0.0002)	(0.0005)	(0.00002)

Figures 15 – 16 display the residuals fit-data versus time and dose. The change in the sign of the estimates in Tables 10 and 11 suggests that the logistic regression models may not be able to predict other data sets well for various lengths of time.

9/10

## 5. Experiment 2

In experiment 2 cells are grown without exposure to toxin. The data appear in Table 11. The number of viable cells is computed using (1.1). The data are displayed in Figure 17, along with the results of fitting a logistic regression model. The regression model appears in Table 12.

**Table 11**  
Cell growth without exposure to toxin

Time (hrs)	ABS for each replicate		
	1	2	3
0	0.071	0.062	0.069
14	0.091	0.120	0.114
24	0.196	0.246	0.192
38	0.264	0.302	0.267
48	0.524	0.631	0.552
62	0.509	0.493	0.513
72	0.086	0.778	0.791
86	0.827	0.946	0.924
96	0.988	1.025	1.101
120	1.108	1.095	1.243
144	1.301	1.059	1.195

# of viable cells =  $(1.19 \times 10^5) + (4.59 \times 10^6 \times \text{ABS}) + (6.18 \times 10^5 \times \text{ABS}^2)$

**Table 12**  
Experiment 2  
Cell growth without exposure to toxin

$$\text{Model: } E[\# \text{ viable cells}] = (7.1 \times 10^6) \frac{\exp\{\beta_0 + (\beta_1 \times \text{Time})\}}{1 + \exp\{\beta_0 + (\beta_1 \times \text{Time})\}}$$

Parameter Estimates (Standard Errors)	
$\beta_0$	$\beta_1$
-2.63	0.037
(0.0036)	( $4.9 \times 10^{-6}$ )



**Table 14**  
Experiments 3 – 6 with controls from Experiments 3 and 5

$$\text{Model: } E[\# \text{ viable cells}] = \frac{(5.5 \times 10^6) \exp\{\chi\beta\}}{1 + \exp\{\chi\beta\}} \text{ where}$$

$$\chi\beta = \beta_0 + (\beta_1 \times \text{Time}) + (\beta_2 \times \text{Dose}) + (\beta_3 \times \text{Dose} \times \text{Time})$$

Parameter Estimates (Standard Errors)			
$\beta_0$	$\beta_1$	$\beta_2$	$\beta_3$
1.65	-0.014	-0.19	-0.01
(0.0004)	(0.00002)	(0.00006)	(4×10 <sup>-6</sup> )

**Table 15**  
Data From Experiment 3  
Confluent plates with no exposure to toxin  
Control data

Time (hrs)	ABS for each replicate			
	1	2	3	4
8	0.807	0.876	0.776	0.882
12	0.499	0.521	0.482	0.459
16	0.568	0.417	0.473	0.218
25	0.596	0.616	0.513	0.562

The addition of the control data does not change the model significantly.  
Figure 20 displays the residuals, fit-data, versus dosage.

### References

- Ballantyne, B., Marrs, T., and Turner, P. (eds.) *General and Applied Toxicology*, Stockton Press, New York, NY 1993.
- Morgan, B.J.T. *Analysis of Quantile Response Data*, Chapman & Hall, London, 1992.
- The Math Works, Inc. *MATLAB Reference Guide*, Version 4.0, The Math Works, Inc. Natick, MA 1992.

EXP 6; TIME=3

o=NUMBER OF VIABLE CELLS; +=FITTED EXPECTED NO.

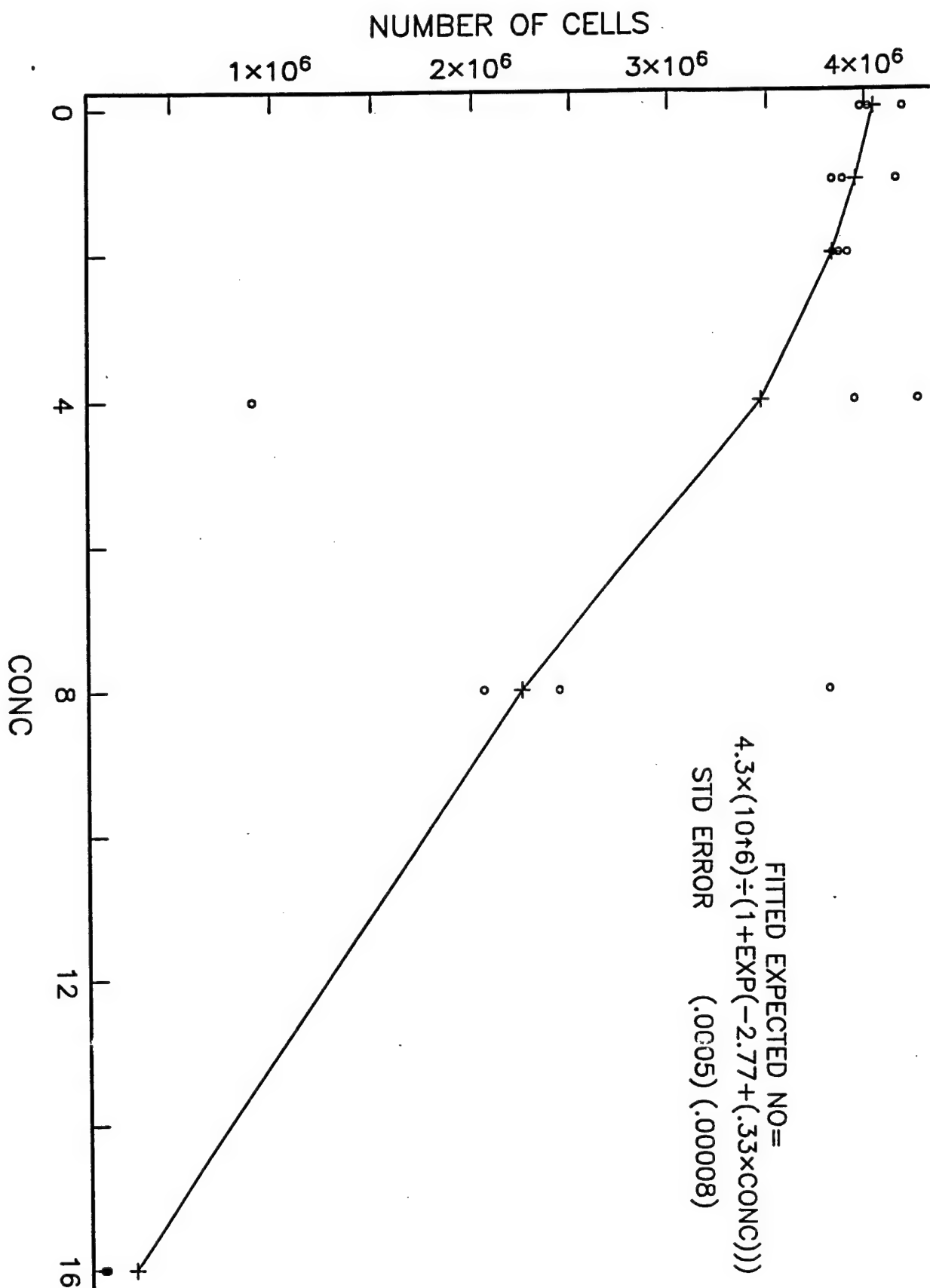


Figure 1

EXP 6; TIME=3; WITHOUT LOW POINT AT CONC 4

o=NUMBER OF VIABLE CELLS; +=FITTED EXPECTED NO.

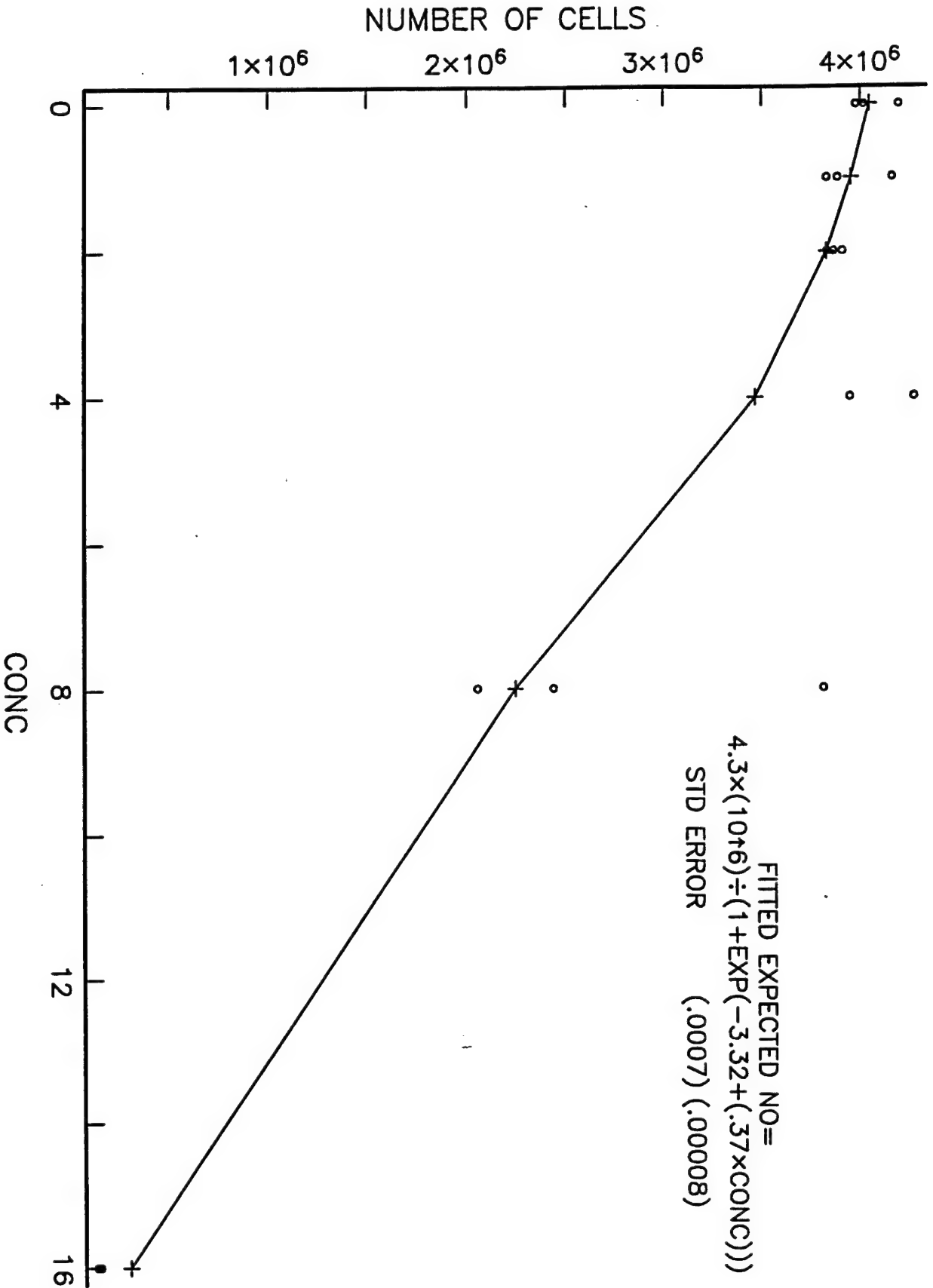


Figure 2

EXP 4; TIME=24

o=NUMBER OF VIABLE CELLS; +=FITTED EXPECTED NO.

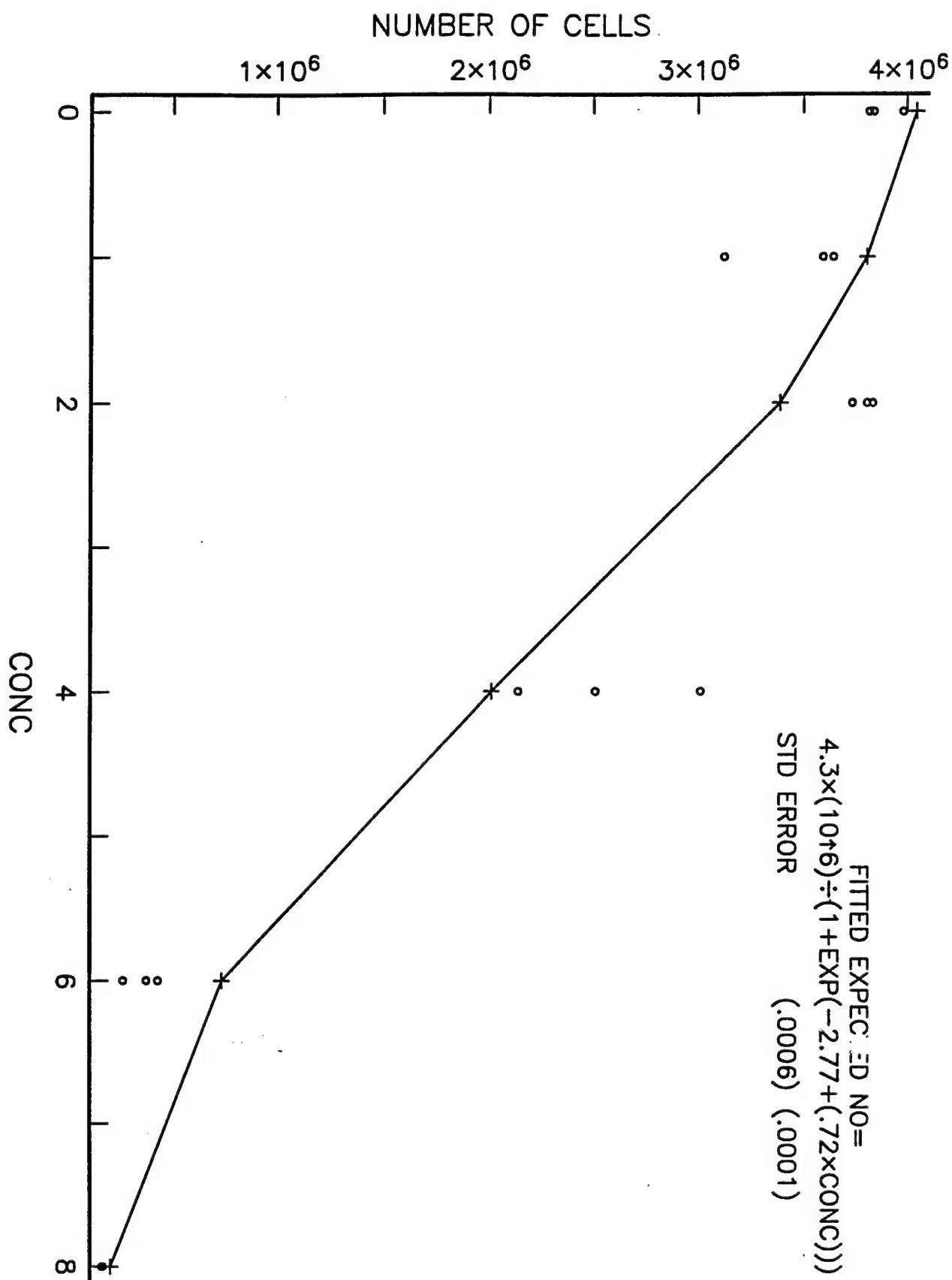


Figure 3

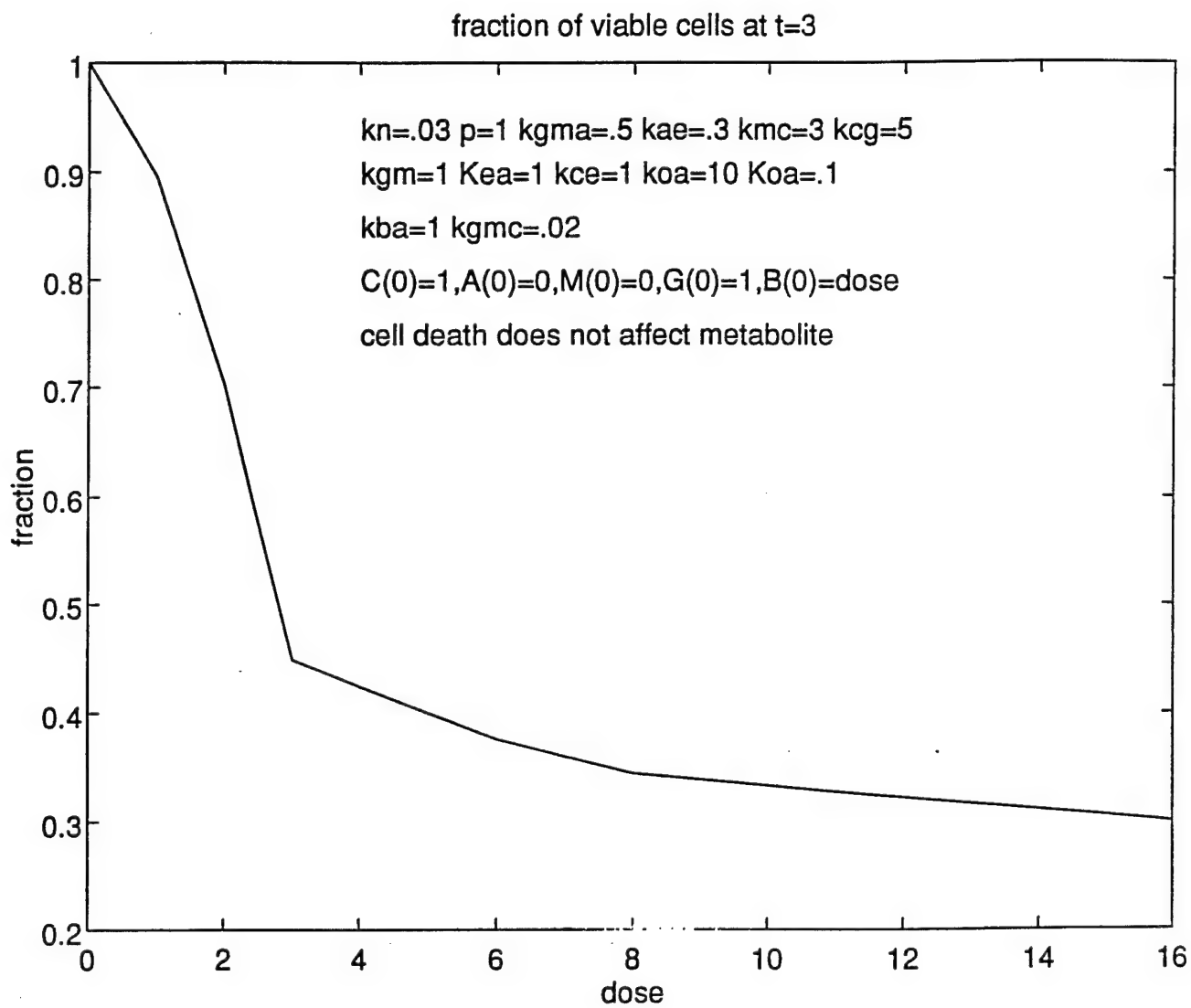


Figure 4

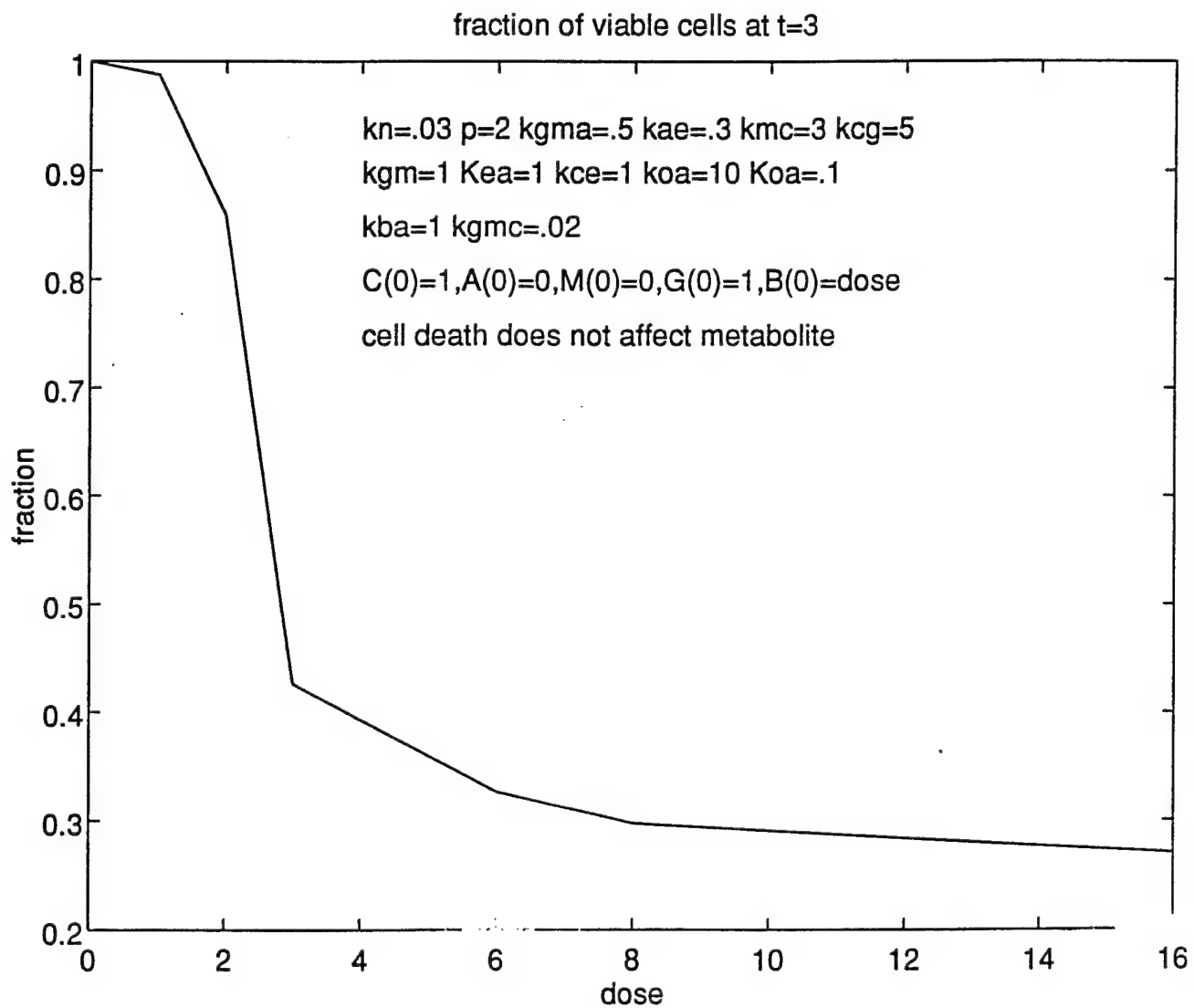


Figure 5

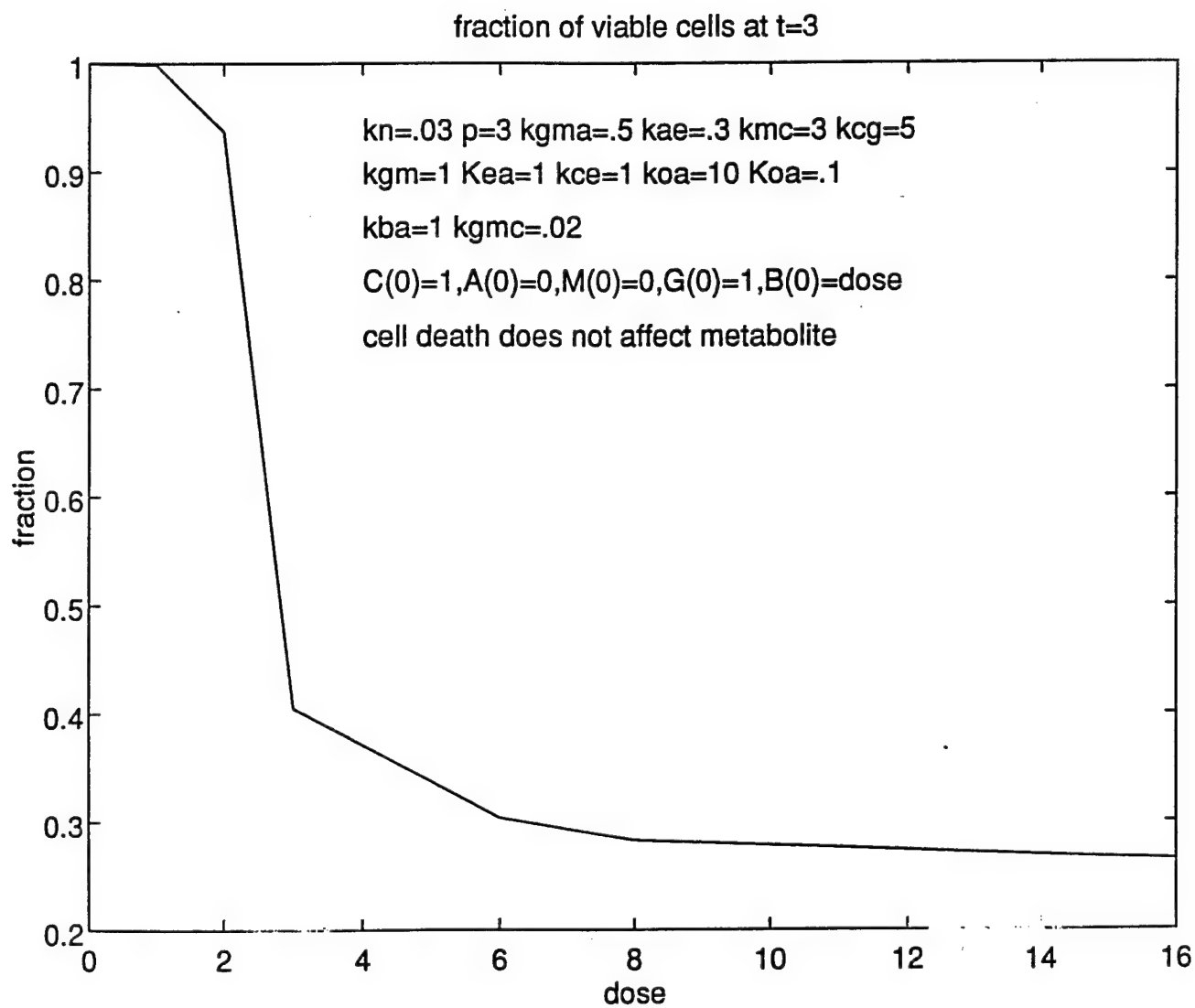


Figure 6

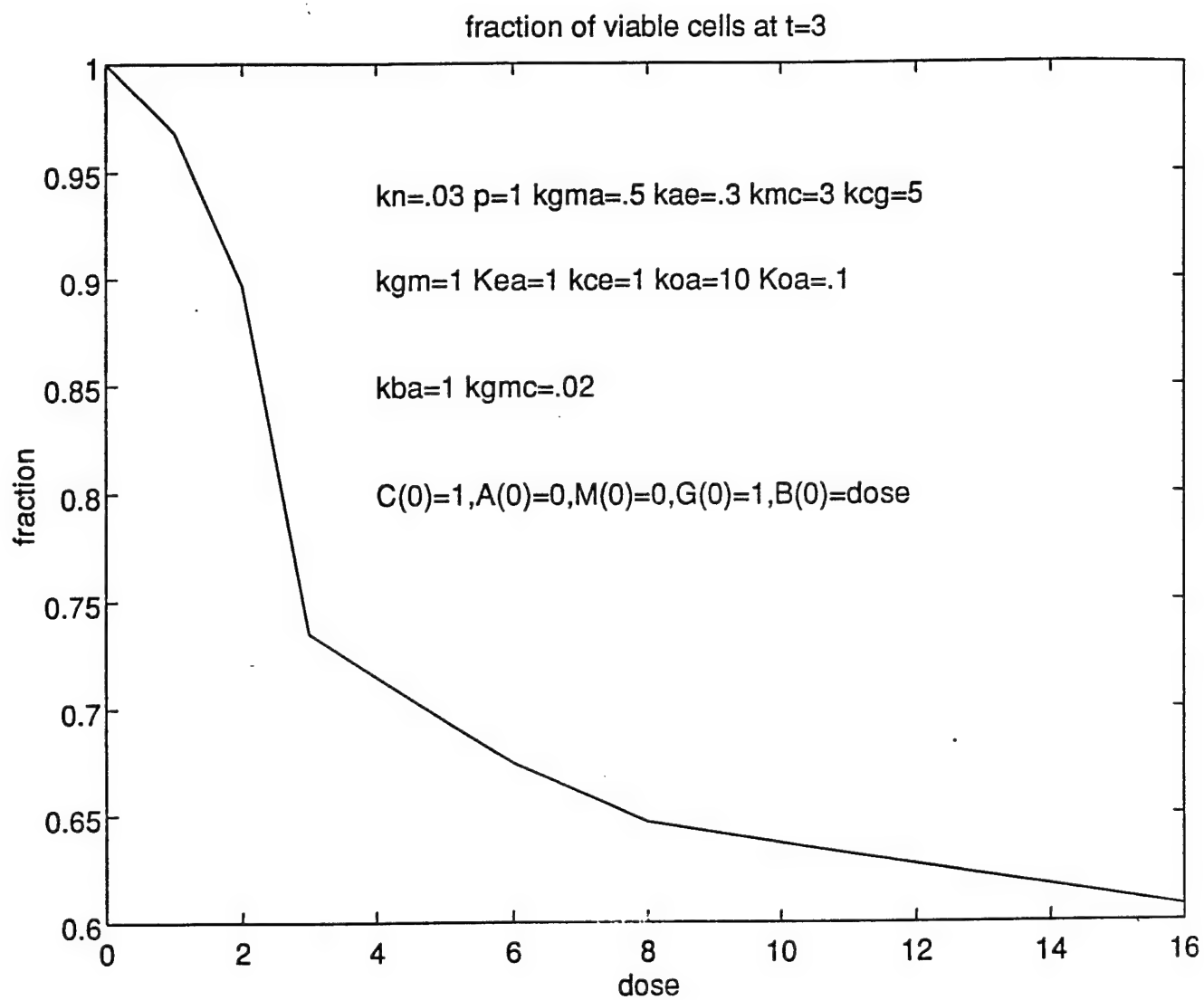


Figure 7



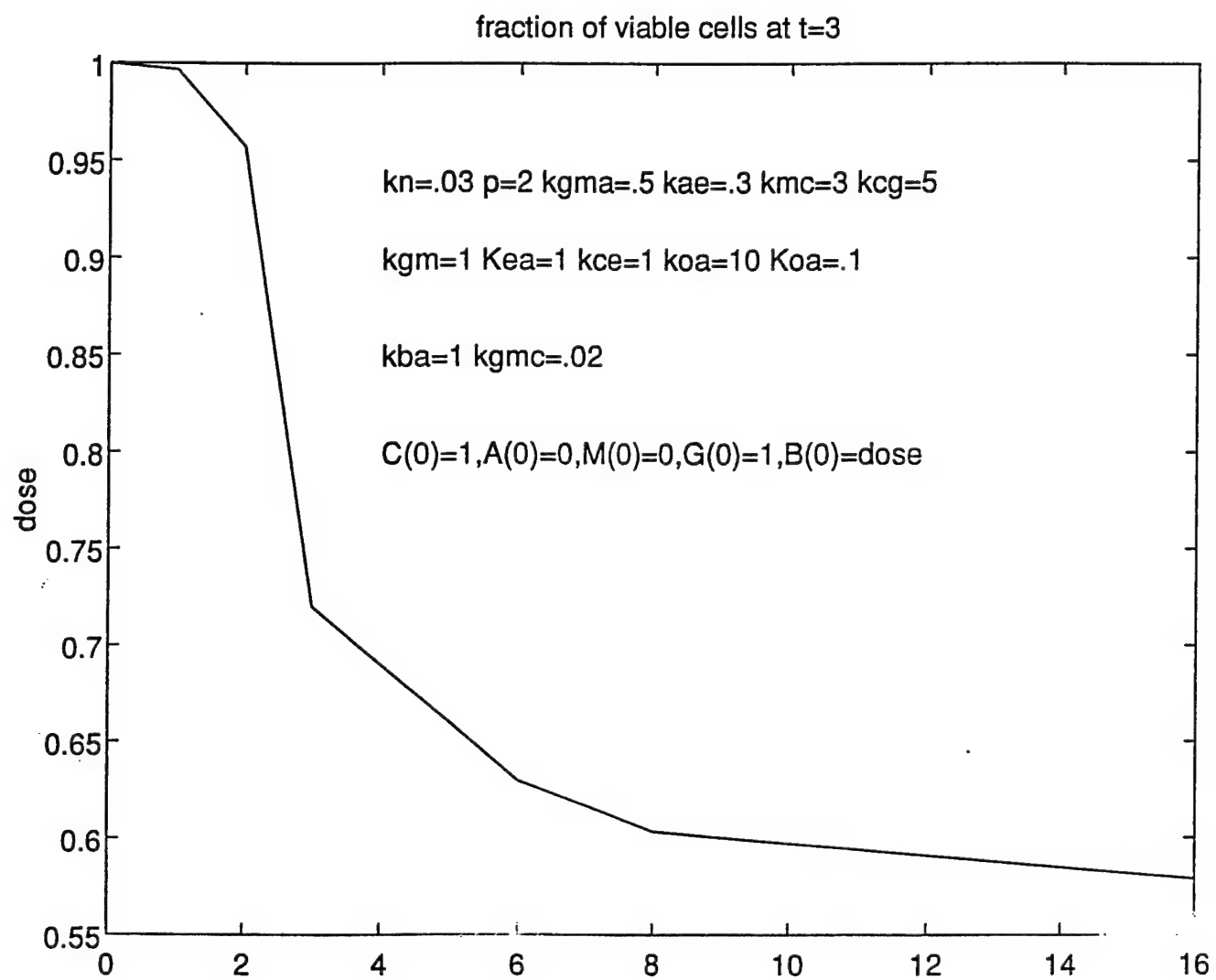


Figure 8

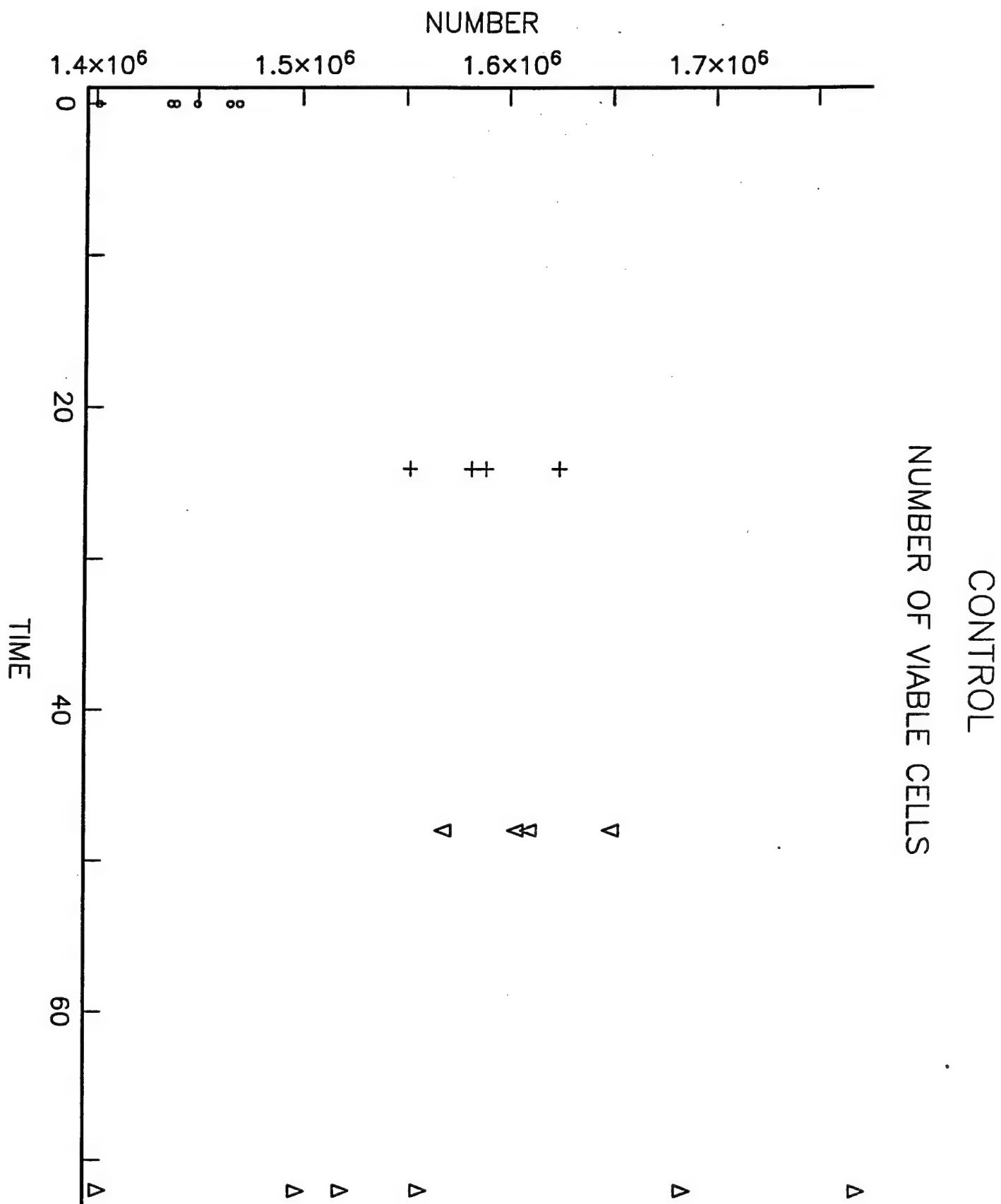


Figure 9

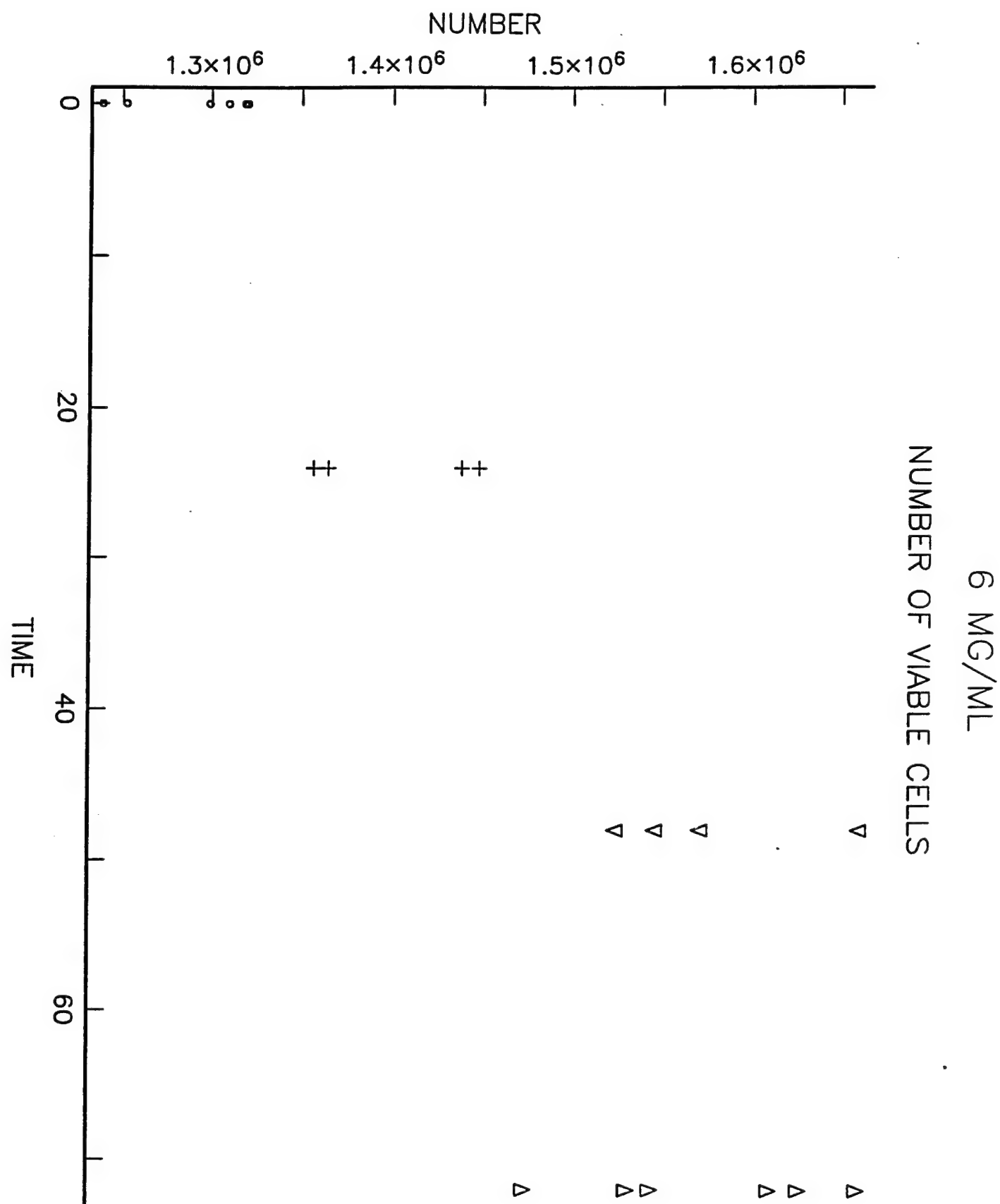


Figure 10

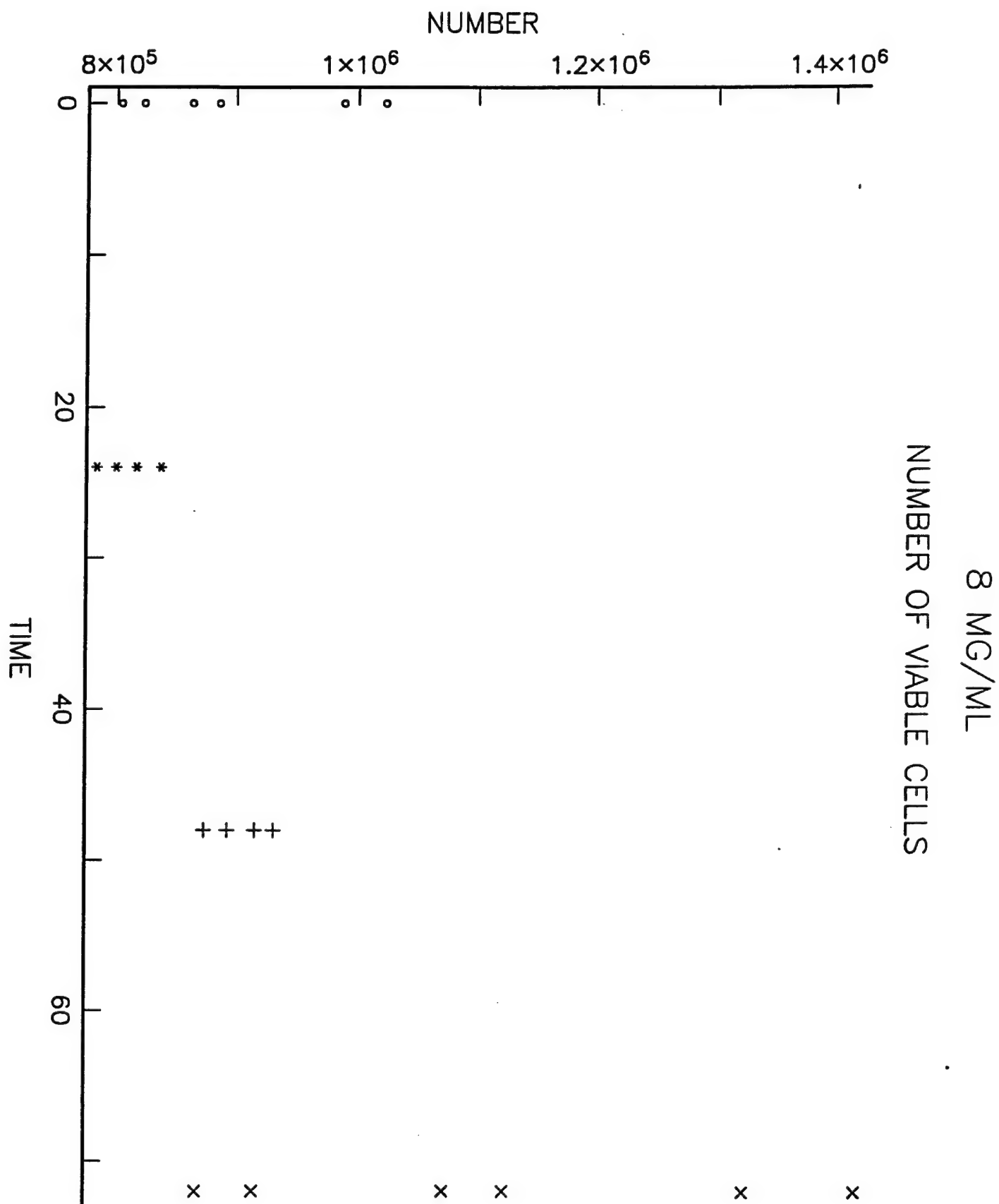


Figure 11

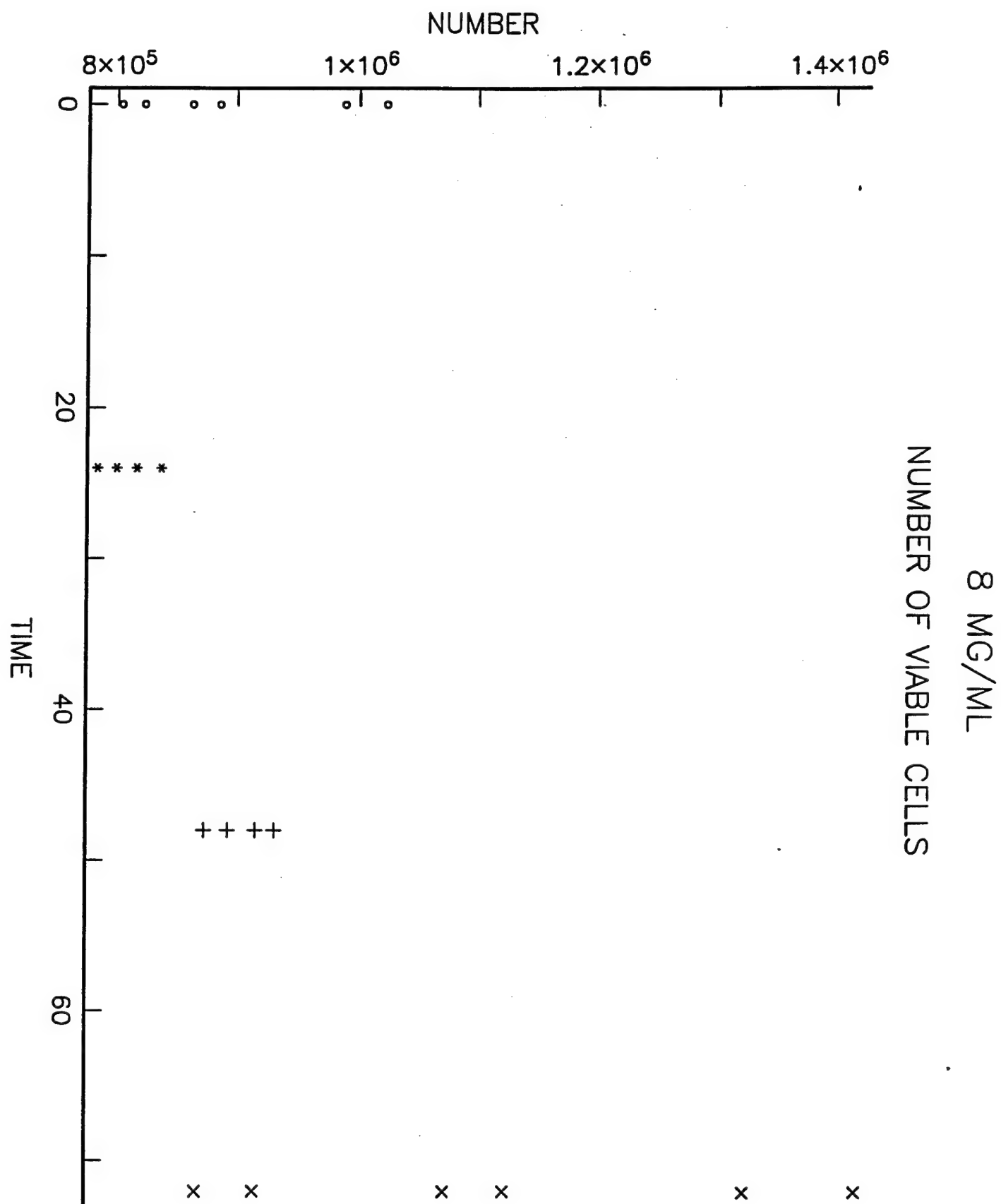


Figure 11

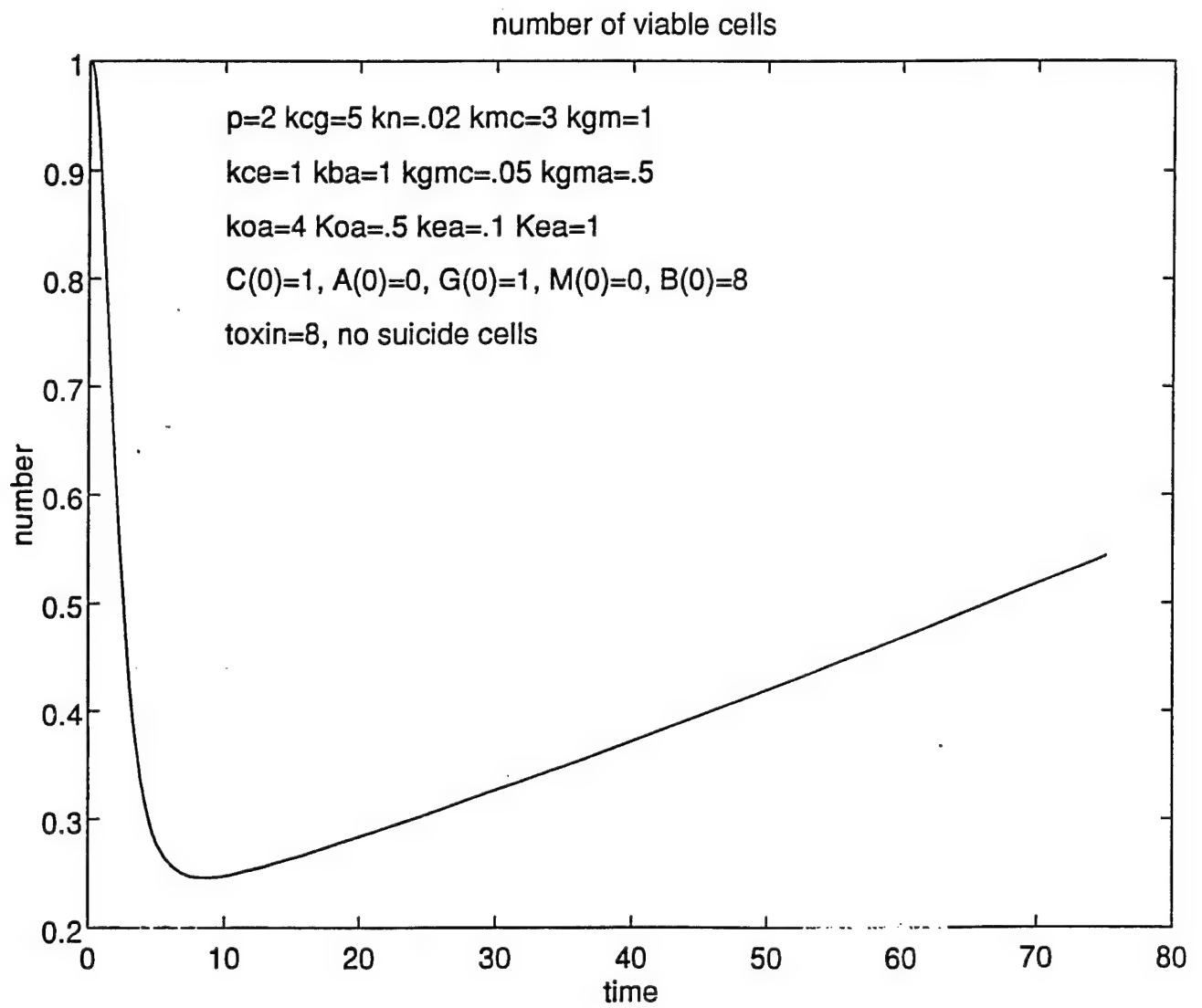


Figure 11a

# EXP3:NUMBER OF CELLS

$$E[\text{NO. CELLS}] = \text{MAXX} \times (\text{EXP}[B_0 + (B_1 \times T)]) \div (1 + \text{EXP}[B_0 + (B_1 \times T)])$$

B0=4.22    B1=-0.29  
 STD ERROR: (.0009)    (.00007)

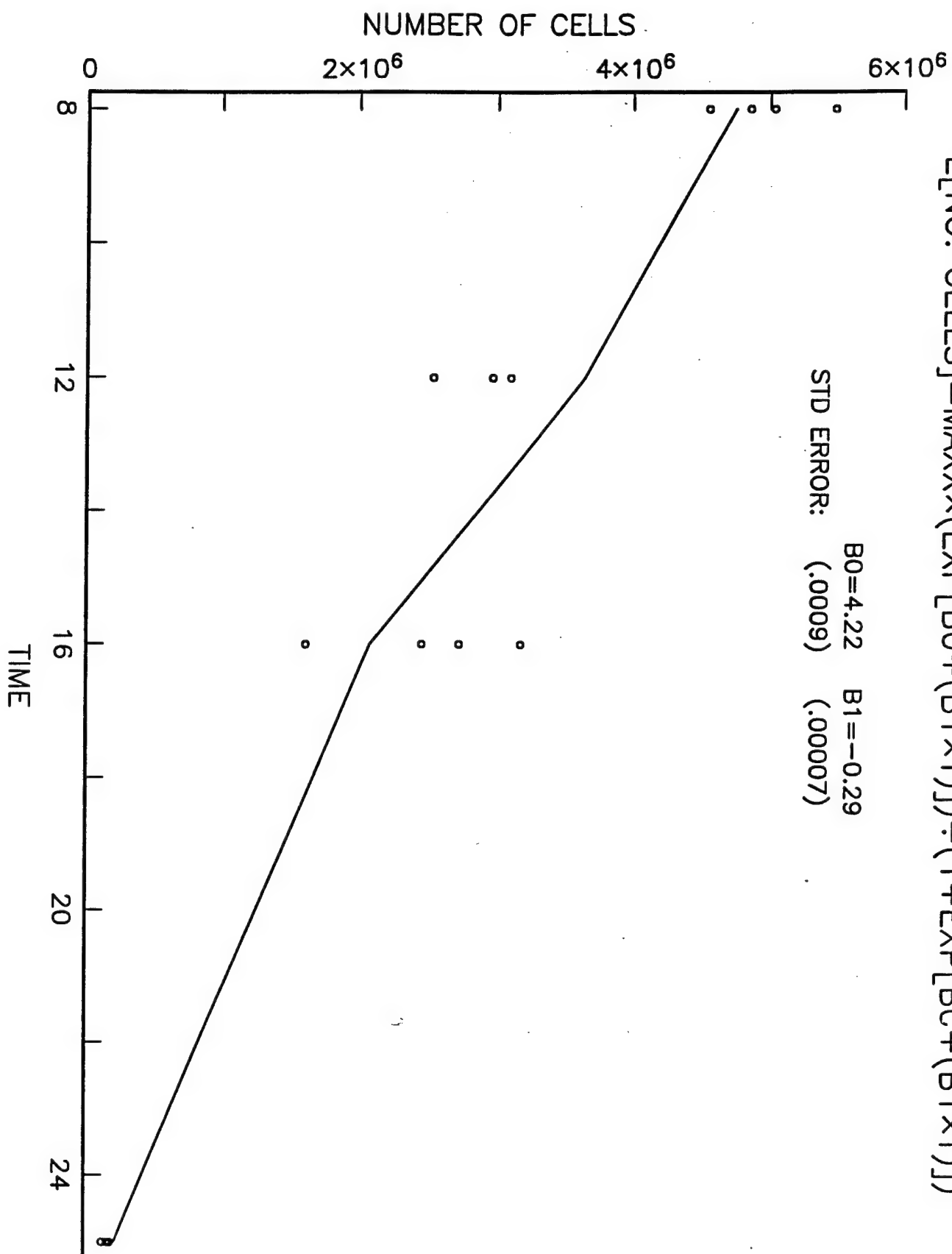


Figure 12

# EXP5:NUMBER OF CELLS

$$E[\text{NO. CELLS}] = \text{MAXX} \times (\text{EXP}[B_0 + (B_1 \times T)]) \div (1 + \text{EXP}[B_0 + (B_1 \times T)])$$

B0=2.15    B1=-0.606  
 STD ERROR: (.0008)    (.0002)

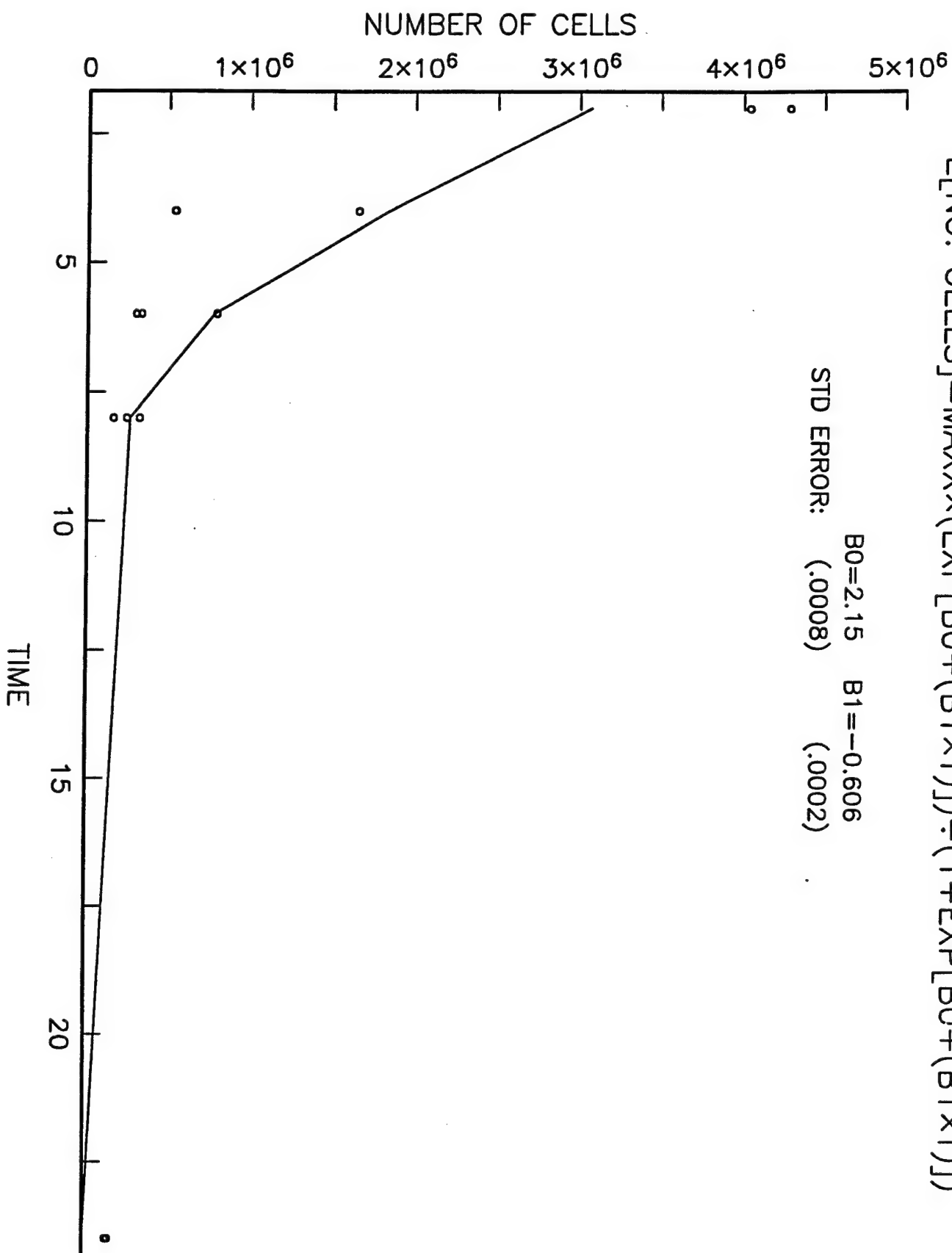


Figure 13



# EXP 3 AND 5

$$E[\text{NO. CELLS}] = \text{MAX} \times \text{EXP}[\text{BX}] \div (1 + \text{EXP}[\text{BX}])$$

$$\text{WHERE } \text{BX} = \text{B0} + (\text{B1} \times \text{TIME}) + (\text{B2} \times \text{DOSE}) + (\text{B3} \times \text{DOSE} \times \text{TIME})$$

$\circ$  STD ERROR: (0.004) (0.0006) (0.0006) (0.00009)  
 $\circ$  B0=12.88 B1=0.39 B2=-1.44 B3=-0.11

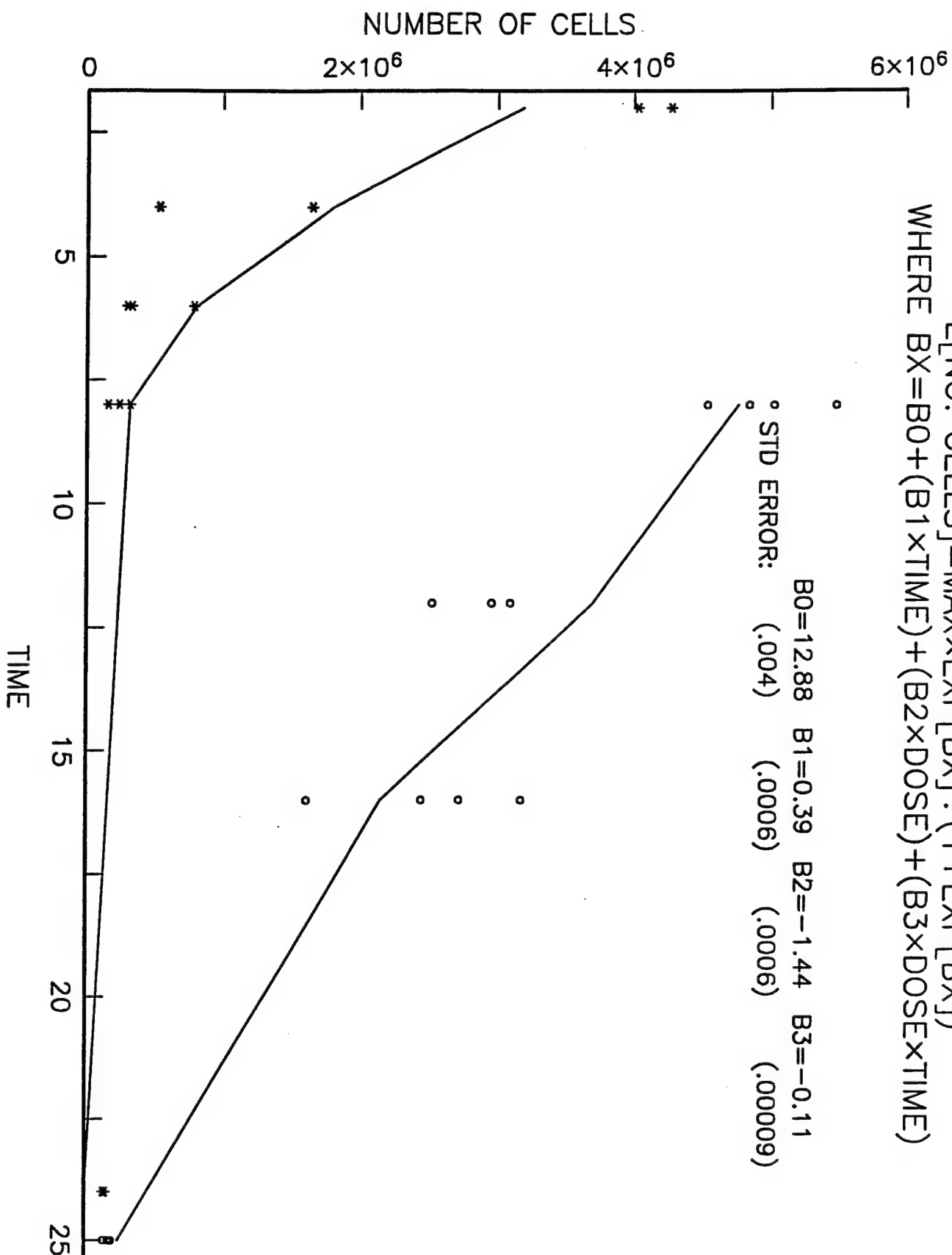


Figure 14

# DATA FROM EXPERIMENTS 3 AND 5 WITH CONTROL FROM 5

## LOGISTIC REGRESSION: FIT-DATA

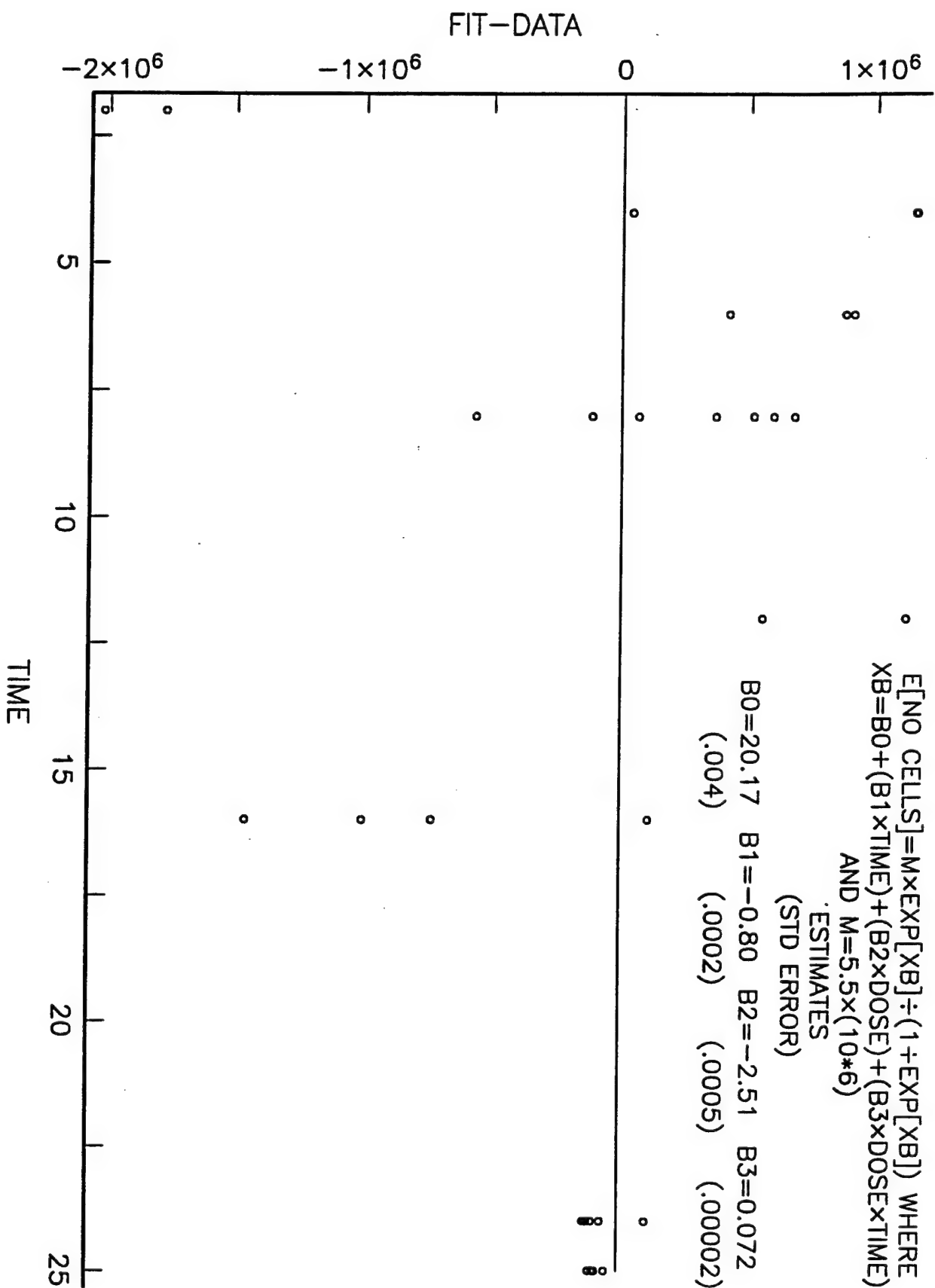


Figure 15

# DATA FROM EXPERIMENTS 3 AND 5 WITH CONTROL FROM 5

## LOGISTIC REGRESSION:FIT-DATA

E[NO CELLS]=M×EXP[XB]÷(1+EXP[XB]) WHERE  
 $XB=B_0+(B_1 \times TIME)+(B_2 \times DOSE)+(B_3 \times DOSE \times TIME)$   
 AND  $M=5.5 \times (10^6)$

ESTIMATES  
 (STD ERROR)

$B_0=20.17$   $B_1=-0.80$   $B_2=-2.51$   $B_3=0.072$   
 (.004) (.0002) (.0005) (.00002)

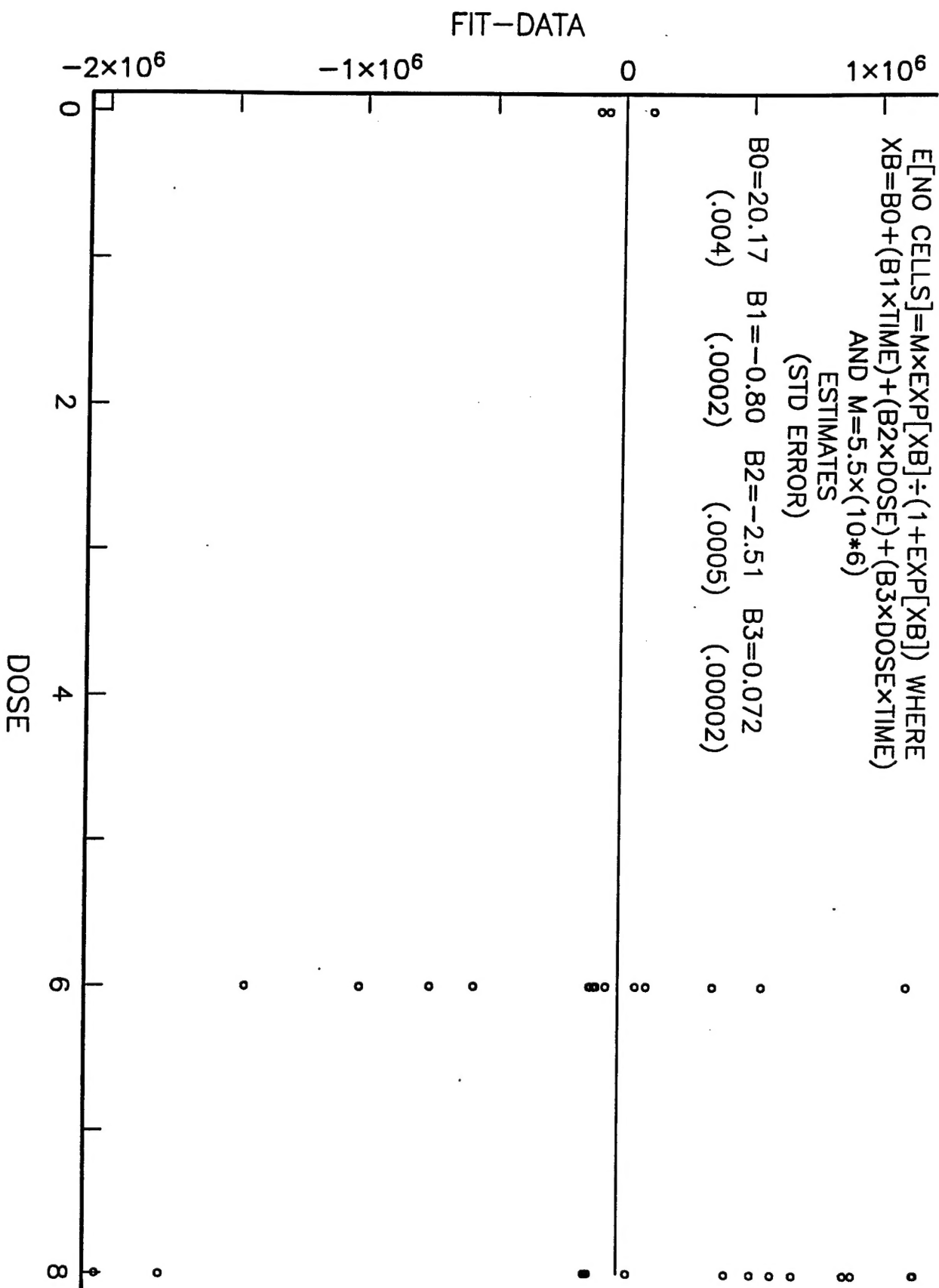


Figure 16

# EXP 2

$$E[\text{NO. CELLS}] = \text{EXP}(B0 + (B1 \times T)) \div (1 + \text{EXP}(B0 + (B1 \times T)))$$

B0 = -2.63

B1 = .037

STD ERROR: (.0036) (4.9x(10\*-6))

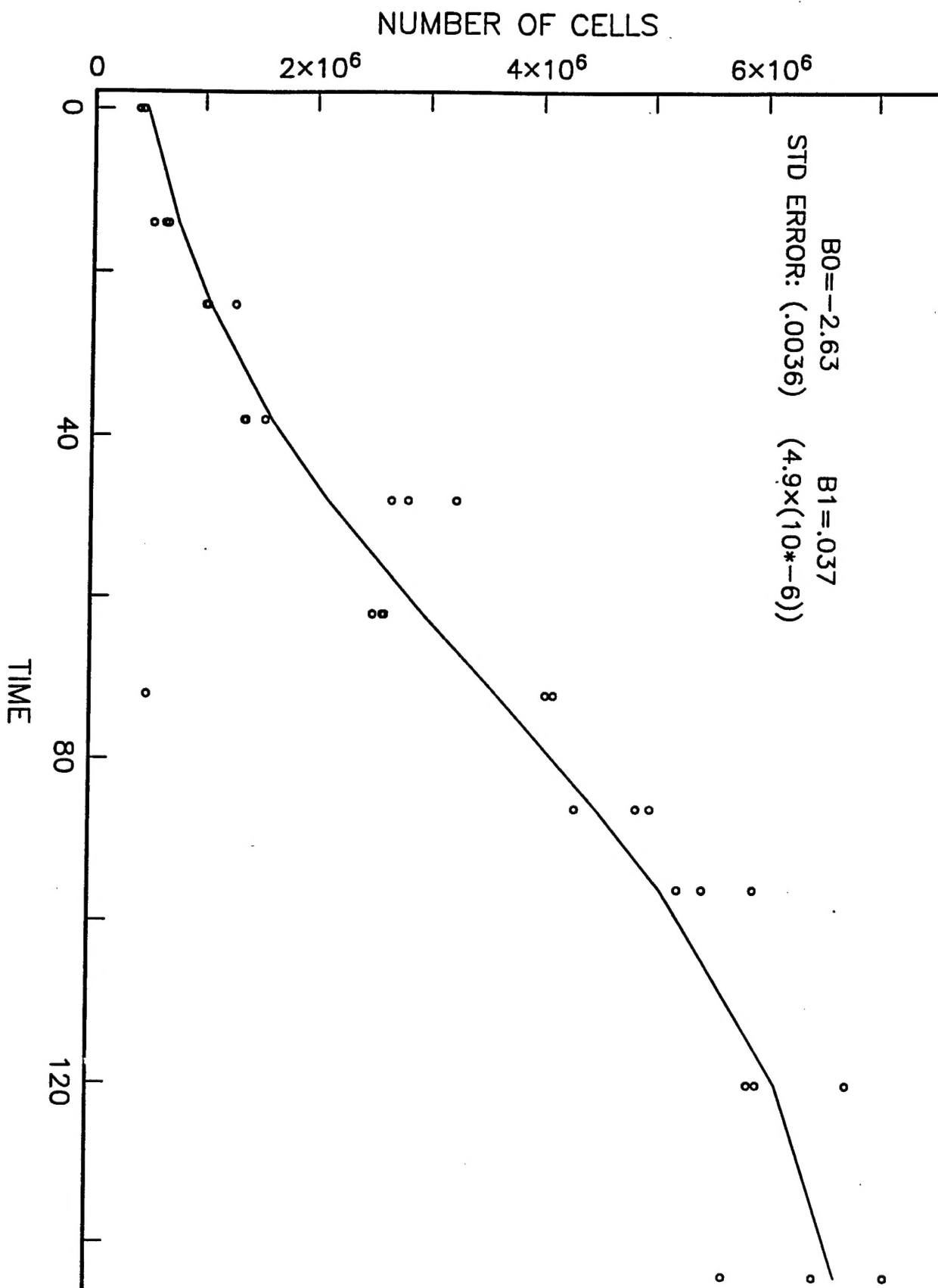


Figure 17

# EXPERIMENTS 3-6 WITH CONTROL FROM 5

## LOGISTIC REGRESSION: FIT-DATA

$E[\text{NO CELLS}] = M \times \exp[XB] \div (1 + \exp[XB])$  WHERE  
 $XB = B_0 + (B_1 \times \text{TIME}) + (B_2 \times \text{DOSE}) + (B_3 \times \text{DOSE} \times \text{TIME})$

AND  $M = 5.5 \times (10^6)$

ESTIMATES

(STD ERROR)

$B_0 = 1.65$   $B_1 = -0.014$   $B_2 = -0.19$   $B_3 = -0.01$   
 (0.0004) (0.00002) (0.00006)  $(4 \times (10^{-6}))$

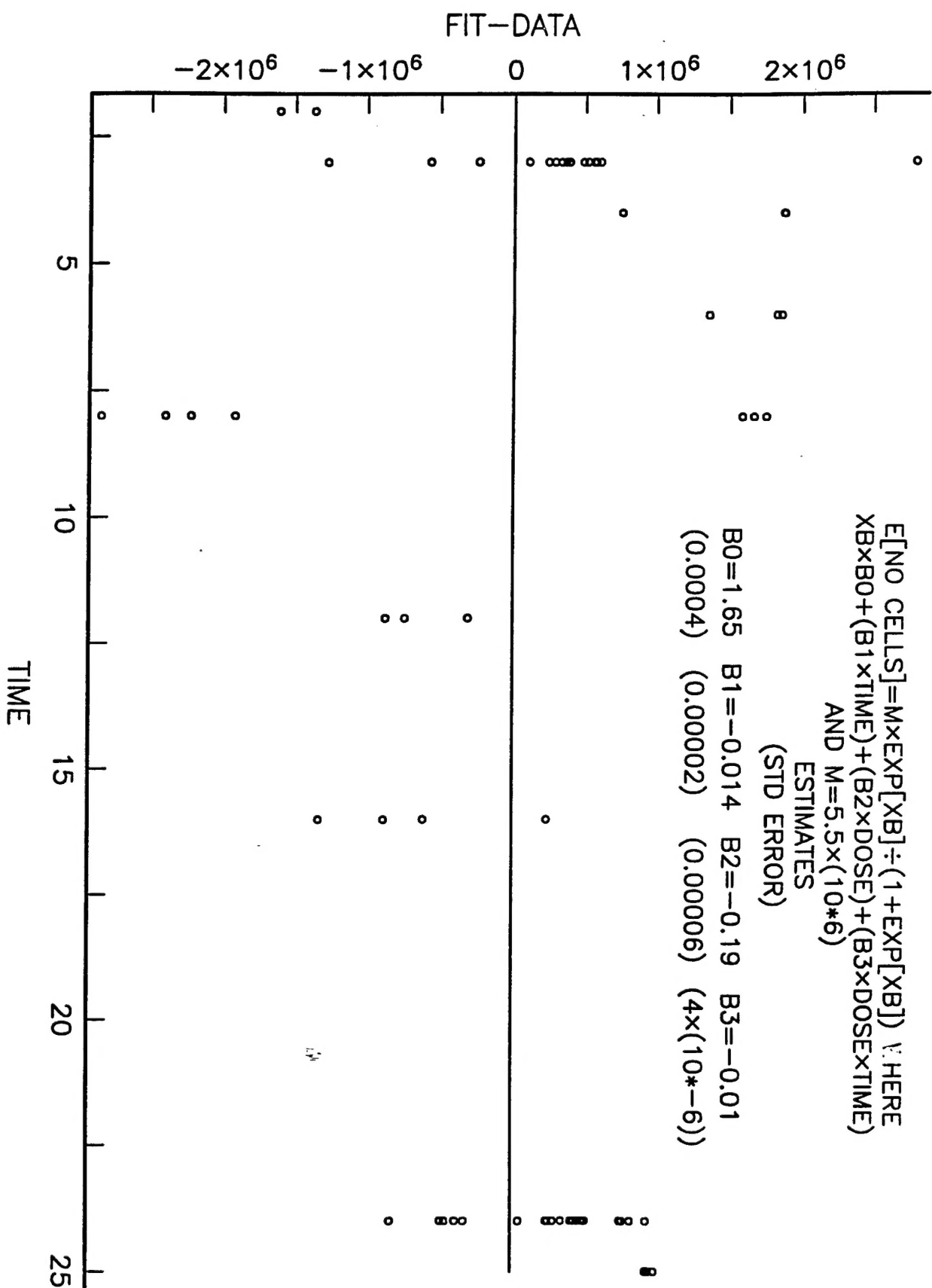


Figure 18

# EXPERIMENTS 3-6 WITH CONTROL FROM 5

## LOGISTIC REGRESSION: FIT-DATA

$E[\text{NO CELLS}] = M \times \text{EXP}[XB] \div (1 + \text{EXP}[XB])$  WHERE  
 $XB = B_0 + (B_1 \times \text{TIME}) + (B_2 \times \text{DOSE}) + (B_3 \times \text{DOSE} \times \text{TIME})$   
 AND  $M = 5.5 \times (10^6)$

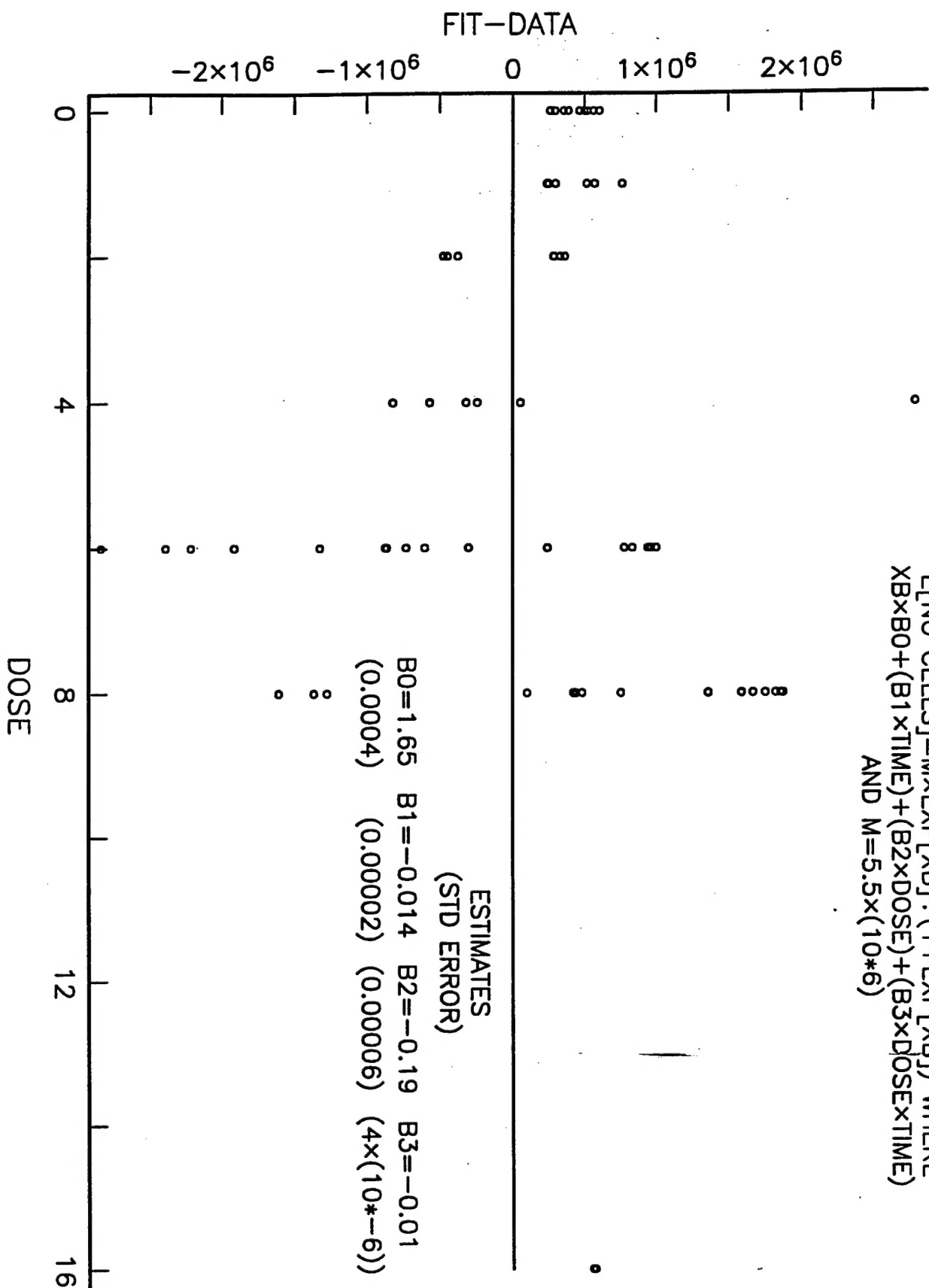


Figure 19